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THE SPECIFICITY OF PEPSIN

BY JOSEPH S. FRUTON AND MAX BERGMANN

WITH THE COLLABORATION OF WILLIAM P. ANSLOW, JR.

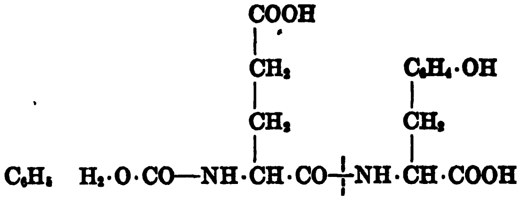
(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, December 13, 1938)

All the hitherto available information regarding the enzymatic action of pepsin has been obtained through the use of proteins as substrates. Much of this information must be regarded as tentative as long as no substrate of simple, well known structure can be provided for the study of pepsin action. It is the purpose of the present communication to report the finding of such substrates for swine pepsin.

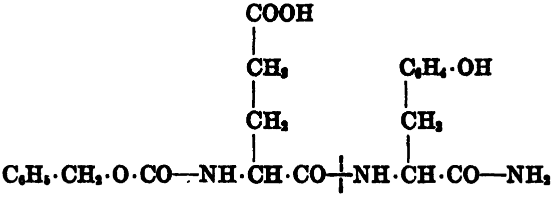
The compound carbobenzoxy-*l*-glutamyl-*l*-tyrosine (I) is hydrolyzed to carbobenzoxyglutamic acid and tyrosine in the presence of crystalline swine pepsin. The hydrolysis occurs optimally at about pH 4 (Fig. 1). At pH 1.8 to 2, which is generally accepted as the optimum for pepsin, the hydrolysis of substrate (I) occurs rather slowly. Repeated recrystallization of the pepsin preparation did not appreciably alter its activity towards substrate (I). On the other hand, inactivation of pepsin at pH 8.0, followed by readjustment of the activity to pH 4.0, resulted in loss of the hydrolytic activity toward the substrate. The possibility that the splitting of substrate (I) by crystalline pepsin might be due to the presence of another enzyme of the cathepsin type is unlikely, since neither cysteine nor hydrogen peroxide had any effect on the rate of hydrolysis. Furthermore, there was no demonstrable carboxypeptidase activity in the enzyme preparations employed. The results of all these experiments justify the conclusion that the splitting of carbobenzoxyglutamyltyrosine is attributable to the action of pepsin itself (Table I).

The specificity of the pepsin action at pH 4 was investigated by means of other synthetic compounds differing from carbobenzoxyglutamyltyrosine to a greater or lesser degree. Substitution of the tyrosine portion of the peptide by various amino acids showed that while carbobenzoxy-*l*-glutamyl-*l*-phenylalanine



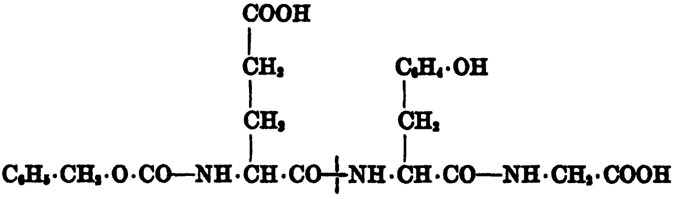
$$C_6H_5\quad H_2\cdot O\cdot CO—NH\cdot\overset{\begin{array}{c}COOH\\|\\CH_2\\|\\CH_2\\|\end{array}}{CH}\cdot CO\,|\,NH\cdot\overset{\begin{array}{c}C_6H_4\cdot OH\\|\\CH_2\\|\end{array}}{CH}\cdot COOH$$

(I)



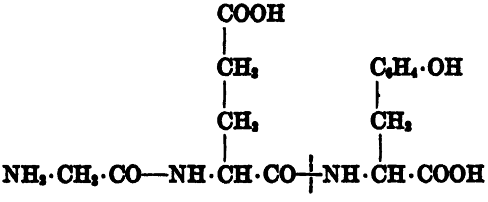
$$C_6H_5\cdot CH_2\cdot O\cdot CO—NH\cdot\overset{\begin{array}{c}COOH\\|\\CH_2\\|\\CH_2\\|\end{array}}{CH}\cdot CO\,|\,NH\cdot\overset{\begin{array}{c}C_6H_4\cdot OH\\|\\CH_2\\|\end{array}}{CH}\cdot CO—NH_2$$

(II)



$$C_6H_5\cdot CH_2\cdot O\cdot CO—NH\cdot\overset{\begin{array}{c}COOH\\|\\CH_2\\|\\CH_2\\|\end{array}}{CH}\cdot CO\,|\,NH\cdot\overset{\begin{array}{c}C_6H_4\cdot OH\\|\\CH_2\\|\end{array}}{CH}\cdot CO—NH\cdot CH_2\cdot COOH$$

(III)



$$NH_2\cdot CH_2\cdot CO—NH\cdot\overset{\begin{array}{c}COOH\\|\\CH_2\\|\\CH_2\\|\end{array}}{CH}\cdot CO\,|\,NH\cdot\overset{\begin{array}{c}C_6H_4\cdot OH\\|\\CH_2\\|\end{array}}{CH}\cdot COOH$$

(IV)

was split fairly rapidly by pepsin with an optimum at about pH 4.5 (*cf.* Fig. 1), carbobenzoxy-*l*-glutamyl-*l*-diiodotyrosine, carbobenzoxy-*l*-glutamyl-*l*-glutamic acid, and carbobenzoxy-*l*-glutamyl-

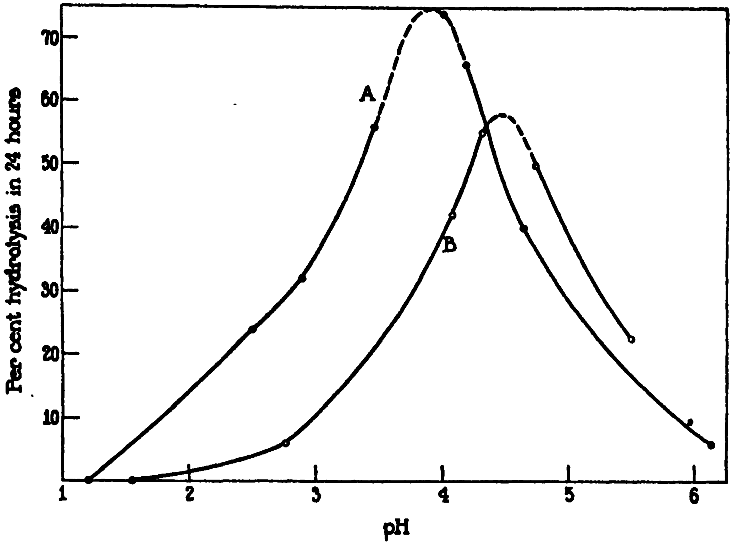


FIG. 1. pH dependence of hydrolysis of synthetic substrates by crystalline pepsin. Curve A, carbobenzoxy-*l*-glutamyl-*l*-tyrosine; Curve B, carbobenzoxy-*l*-glutamyl-*l*-phenylalanine. Enzyme concentration, 2.4 mg. of pepsin N per cc.

TABLE I

*Hydrolysis of Carbobenzoxyglutamyltyrosine with Crystalline Pepsin**

Enzyme preparation	Time	Hydrolysis
	hrs.	*per cent*
Twice crystallized pepsin, 1.4 mg. protein N per cc.	24	53
" " " inactivated at pH 8 and readjusted to pH 4	24	0
4 times crystallized pepsin, 1.5 mg. protein N per cc.	24	54
Twice crystallized pepsin, 1.4 mg. protein N per cc.	22.5	51
	46.5	72
" " " + cysteine (0.005 mM per cc.)	22.5	53
	46.5	72
Twice crystallized pepsin, 0.8 mg. protein N per cc.	24	33
" " " + hydrogen peroxide (0.2 mM per cc.)	24	35

* pH 4.0.

glycine were resistant to pepsin action (Table II). On the other hand, substitution of the glutamic acid residue indicated that both carbobenzoxyglycyl-*l*-tyrosine and carbobenzoxy-*l*-tyrosyl-*l*-tyrosine were split by pepsin at a rather slow rate. All the peptides which were found in these experiments to be hydrolyzed by swine pepsin contain the aromatic amino acid residues tyrosine or phenylalanine. In the most sensitive substrates, tyrosine is combined with glutamic acid. On the basis of these experiments one may infer that the sensitivity of a peptide bond toward pepsin depends upon the nature of both amino acid residues which participate in the peptide bond that is hydrolyzed. However, there

TABLE II

*Behavior of Synthetic Substrates toward Crystalline Pepsin**

Substrate	Time	Hydrolysis	Isolation of products
	hrs.	*per cent*	
Carbobenzoxy-*l*-glutamyl-*l*-tyrosine	6	18	Carbobenzoxy-*l*-glutamic acid
	24	64	
	48	81†	Tyrosine
Carbobenzoxy-*l*-glutamyl-*l*-phenylalanine	24	26	
	48	51	
	96	93	
Carbobenzoxy-*l*-glutamyl-*l*-diiodotyrosine	24	0	
	48	−1	
Carbobenzoxy-*l*-glutamyl-*l*-glutamic acid	24	3	
	48	3	
	96	5	
Carbobenzoxy-*l*-glutamylglycine	24	2	
	48	3	
	96	5	
Carbobenzoxy-*l*-tyrosyl-*l*-tyrosine	24	8	
	48	16	
	96	29	
Carbobenzoxyglycyl-*l*-tyrosine	24	11	
	48	30	
	96	43	

* pH 4.0; 1.6 mg. of pepsin N per cc.

† Beginning of tyrosine crystallization. If the reaction is allowed to proceed, about 50 per cent of the liberated tyrosine crystallizes out.

are still other structural details of the substrate molecule which influence its sensitivity towards pepsin.

All the previously mentioned synthetic substrates for pepsin contain a free α-carboxyl and a free γ-carboxyl close to the peptide bond which is hydrolyzed by the enzyme. Substitution of the α-carboxyl of substrate (I) to yield carbobenzoxy-*l*-glutamyl-*l*-tyrosineamide (II) strongly depresses the rate of hydrolysis but does not eliminate it entirely. However, if both carboxyls are masked to give carbobenzoxy-*l*-glutaminyl-*l*-tyrosineamide, the product is entirely resistant to pepsin action (Table III). Furthermore, substitution of only the γ-carboxyl, as in carbobenzoxy-*l*-glutaminyl-*l*-phenylalanine, results in a slowing down of the hydrolysis, when compared with the hydrolysis of the acid containing two carboxyls (Table IV). One may conclude, therefore, that the presence of free carboxyls is favorable to the action

TABLE III

*Behavior of Synthetic Substrates toward Crystalline Pepsin**

Substrate	Time	Hydrolysis	Isolation of products
	hrs.	*per cent*	
Carbobenzoxy-*l*-glutamyl-*l*-tyrosine	24	53	
	48	74	
Carbobenzoxy-*l*-glutamyl-*l*-tyrosineamide	43	11	
	68	25	
Carbobenzoxy-*l*-glutaminyl-*l*-tyrosineamide	43	3	
	68	5	
Carbobenzoxy-*l*-glutamyl-*l*-tyrosylglycine	24	39	Carbobenzoxy-*l*-glutamic acid
	48	68	
Carbobenzoxyglycyl-*l*-glutamyl-*l*-tyrosine	24	48	Tyrosine
	48	52†	
l-Glutamyl-*l*-tyrosine	74	3	
Glycyl-*l*-glutamyl-*l*-tyrosine	24	32	
	48	43	
Benzoyl-*l*-tyrosine	48	2	
Chloroacetyl-*l*-tyrosine‡	24	0	

* pH 4.0; 1.4 mg. of pepsin N per cc.

† Tyrosine crystallization.

‡ An experiment at pH 7.0 also showed no hydrolysis of this substrate by the pepsin preparation.

of pepsin if other structural requisites for the enzymatic action are fulfilled. In order to subject this conclusion to still another test, carbobenzoxy-*l*-glutamyl-*l*-tyrosylglycine (III) was investigated. The latter possesses a free α-carboxyl but at a greater distance from the glutamyltyrosine linkage than in (I). However, the rate of peptic hydrolysis of (III) is only slightly diminished when compared with that of (I); it is, however, remarkably increased when compared with that of the amide (II).

These results showing the favorable influence of free carboxyls lead to the expectation that pepsin action is inhibited by the presence, in the substrate, of a free amino group in close proximity to the peptide bond. *l*-Glutamyl-*l*-tyrosine is completely resistant to the action of pepsin. Furthermore, the following compounds containing basic groups were tested for lability toward pepsin, with completely negative results at pH 2 and pH 4: benzoyl-*l*-lysineamide, benzoylglycyl-*l*-lysineamide, benzoyl-*l*-histidineamide, benzoylglycyl-*l*-histidineamide, benzoyl-*l*-arginineamide. However, the free tripeptide glycyl-*l*-glutamyl-*l*-tyrosine (IV) is split by pepsin with remarkable ease (Table III) between the glutamyl and tyrosine residues.

TABLE IV

*Behavior of Synthetic Substrates toward Crystalline Pepsin**

Substrate	Time	Hydrolysis
	hrs.	*per cent*
Carbobenzoxy-*l*-glutamyl-*l*-phenylalanine	24	24
	46	38
Carbobenzoxy-*l*-glutamyl-*d*-phenylalanine	24	5
	46	3
Carbobenzoxy-*l*-glutaminyl-*l*-phenylalanine	20	8
	44	26
	92	50
Carbobenzoxy-*l*-phenylalanyl-*l*-glutamic acid	20	6
	44	20

* pH 4.2; 1.4 mg. of pepsin N per cc.

The specificity of pepsin is sensitive not only to the nature of the amino acids which participate in the peptide bond of the substrate, but also to the sequence of these amino acids. As an example, carbobenzoxy-*l*-phenylalanyl-*l*-glutamic acid is hydrolyzed much more slowly than is carbobenzoxy-*l*-glutamyl-*l*-phenylalanine (Table IV).

The optical selectivity of pepsin is demonstrated in the resistance of carbobenzoxy-*l*-glutamyl-*d*-phenylalanine to the hydrolytic action of the enzyme (Table IV).

A few years ago Calvery and Schock (1) found that pepsin liberates tyrosine from egg albumin. It was rather difficult to reconcile this observation with the generally accepted opinion that all polypeptides were resistant to pepsin action. The hypothesis was discussed that, during the pepsin action on egg albumin, there might be formed peptides containing tyrosine in a special, hitherto unknown, and extremely labile combination (2). Hypotheses of this kind now become unnecessary, since it has been shown that pepsin splits peptide linkages of simple peptides, such as glycylglutamyltyrosine, with the liberation of tyrosine. The latter process is comparable to the formation of tyrosine during the pepsin action on egg albumin. Our experiments have demonstrated that pepsin is not restricted to the splitting of centrally located peptide bonds but also hydrolyzes terminal peptide bonds if they are situated at the carboxyl end of the peptide chain. The formation of tyrosine during the peptic digestion of a genuine protein probably proceeds in such a manner that at first the carboxyl group of tyrosine is liberated and that afterwards the peptide bond involving the amino group of tyrosine is hydrolyzed.

The enzymes that digest genuine proteins are usually classified as pepsinases, tryptases, or papainases on the basis of the pH optimum of their rather complex action on proteins (3). It is assumed that pepsin has its optimum around pH 1.8,[1] while the optimum of the papainases was found to depend upon the nature of the substrates and to vary from pH 3 to pH 10. However, the experiments with synthetic substrates have demonstrated the pepsin action to depend largely upon the structure of the substrate and to extend deeply into the range which was hitherto regarded as being reserved for the papainases. Consequently, it is not possible any longer to base the differentiation between pepsin and papainases on the assumption that pepsin has a single characteristic pH optimum. Another consequence is the following. If it is desired to investigate whether a given peptide or peptide derivative can be hydrolyzed by pepsin, a series of tests at different pH values must be performed. The splitting of carbobenzoxyglutamylphenylalanine by pepsin would never have been observed if experiments had been performed only at pH 1.8.

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[1] Dyckerhoff and Tewes (4) observed that the splitting of casein and gelatin by pepsin is, under certain conditions, more rapid at pH 4 than at pH 2. However, Northrop (5) has shown that the pH determinations performed by these authors by means of the indicator method were erroneous and that their experiments were actually carried out at about pH 2.35 and not at pH 4.

The preference shown by pepsin to peptides containing glutamic acid[3] in addition to tyrosine or phenylalanine may partly explain the rapid and extensive hydrolysis by pepsin of proteins such as edestin, casein, and egg albumin (6) which contain many aminodicarboxylic acid residues as well as tyrosine and phenylalanine. The slow peptic hydrolysis of gelatin (6) corresponds to a very low content of aromatic amino acids. Protamines with a very high content of basic amino acids are either resistant to pepsin or attacked very slowly by the enzyme. This is in accord with the fact that no peptide derivative of well established structure and containing a basic amino acid as constituent is known to be digested by pepsin. Before a final conclusion is reached, however, it seems desirable to study a greater number of peptides which contain the residues of basic amino acids, and especially those peptides that contain the residues of basic amino acids as well as of aromatic amino acids.

Pepsin is not the only proteinase of the gastrointestinal tract that is specifically adapted to the hydrolysis of substrates containing tyrosine or phenylalanine. Chymotrypsin exhibits a similar preference towards the residues of these aromatic amino acids (7). Nevertheless, both enzymes exhibit distinctly different specificities. For example, pepsin hydrolyzes carbobenzoxyglutamyltyrosine between the two amino acid residues, thus demonstrating that it is not inhibited by an α-carboxyl in close proximity to the peptide bond which is attacked. Chymotrypsin does not hydrolyze the previously mentioned substrate; the enzyme does, however, hydrolyze carbobenzoxytyrosylglycineamide between the tyrosine and the glycine residues. Carbobenzoxytyrosylglycine is not attacked by chymotrypsin. Thus chymotrypsin is, in contrast to swine pepsin, highly sensitive towards the α-carboxyl. However, both enzymes are similar in that they do not require a basic group within their respective substrates. Therefore, the enzymatic hydrolysis of, for example, carbobenzoxyglutamyltyrosine by pepsin represents the interaction of an acidic enzyme and an acidic substrate.

---

[3] The behavior of peptides containing aspartic acid has not been investigated as yet.

The availability of synthetic substrates for pepsin having only one sensitive peptide bond permits a more precise study to be made of the kinetics of peptic hydrolysis and the quantitative estimation of pepsin in biological fluids. Furthermore, it permits a comparative study of the relative specificities of pepsins of various animal species to be undertaken.

The authors wish to express their thanks to Mr. S. Nagy who performed the analyses reported in this paper.

EXPERIMENTAL

Carbobenzoxy-l-Glutamyl-l-Tyrosine[3]—This compound was prepared as described in (8).

The enzymatic hydrolysate by pepsin of 554 mg. of this substance was filtered from a small amount of crystalline material which had separated during the reaction (15 mg. of tyrosine), concentrated to a small volume, and acidified to Congo red. The resulting oil was extracted with ethyl acetate. The ethyl acetate extract was dried and concentrated. Addition of petroleum ether resulted in the crystallization of a material (175 mg.) which after recrystallization from ethyl acetate-petroleum ether melted at 116°. When mixed with an authentic sample of carbobenzoxy-*l*-glutamic acid, the melting point was 118°. The aqueous layer from the ethyl acetate extraction was adjusted to pH 4 and cooled strongly. Tyrosine crystals separated out (32 mg.). Calculated for tyrosine, 7.7 per cent NH_2-N; found, 7.9 per cent NH_2-N.

Carbobenzoxy-l-Glutamyl-l-Phenylalanine

Carbobenzoxy-l-Glutamyl-l-Phenylalanine Ethyl Ester—To an ethyl acetate solution of phenylalanine ethyl ester (prepared from 5 gm. of *l*-phenylalanine) 3.5 gm. of carbobenzoxyglutamic acid anhydride were added to a faintly alkaline reaction. The reaction mixture was allowed to stand for 3 hours, washed with dilute hydrochloric acid and water, and concentrated down. The resulting material was recrystallized from hot ethyl acetate. Yield, 4.2 gm. M.p., 144°.

---

[3] The glutamic acid residue in this compound and in the subsequent glutamyl peptides is linked to the next amino acid through the α-carboxyl group.

$C_{24}H_{28}O_7N_2$. Calculated. C 63.1, H 6.1, N 6.1
456.5 Found. “ 62.8, “ 5.9, “ 6.1

Carbobenzoxy-l-Glutamyl-l-Phenylalanine—1.1 gm. of the above ester were shaken with 5 cc. of N NaOH for 15 minutes. On acidification to Congo red there separated a syrup which quickly crystallized. After recrystallization from ethyl alcohol-water the material melted at 162°. Yield, 0.8 gm.

$C_{22}H_{24}O_7N_2$. Calculated. C 61.7, H 5.6, N 6.5
428.4 Found. “ 61.6, “ 5.7, “ 6.6
$[\alpha]_D^{28} = +12.2°$ (2.4% in N NaOH)

Carbobenzoxy-l-Glutamyl-d-Phenylalanine

Carbobenzoxy-l-Glutamyl-d-Phenylalanine Ethyl Ester—This compound was prepared in the same manner as was the *l* form. M.p., 131°.

$C_{24}H_{28}O_7N_2$. Calculated. C 63.1, H 6.1, N 6.1
456.5 Found. “ 62.8, “ 6.1, “ 6.3

Carbobenzoxy-l-Glutamyl-d-Phenylalanine—This compound was prepared in the same manner as was the *l* form. M.p., 122°.

$C_{22}H_{24}O_7N_2$. Calculated. C 61.7, H 5.6, N 6.5
428.4 Found. “ 61.4, “ 5.7, “ 6.4
$[\alpha]_D^{28} = -21.5°$ (2.4% in N NaOH)

Carbobenzoxy-l-Glutamyl-l-Diiodotyrosine—1.7 gm. of carbobenzoxyglutamyltyrosine were dissolved in 25 cc. of concentrated ammonium hydroxide and 8 cc. of N I_2-KI solution were added dropwise with shaking. On acidification to Congo red there resulted a gelatinous precipitate which was filtered off, washed with water, and dried. The pure diiodo compound (1.4 gm.) was obtained by crystallization from hot alcohol. M.p., 188°.

$C_{22}H_{22}O_8N_2I_2$. Calculated. C 37.9, H 3.2, N 4.0, I 36.5
696.3 Found. “ 38.0, “ 3.3, “ 3.8, “ 36.3

The same substance was also obtained by coupling carbobenzoxyglutamic acid anhydride with diiodotyrosine methyl ester and saponifying the resulting ester.

Carbobenzoxy-l-Glutamyl-l-Tyrosineamide—1 gm. of the corresponding ester (1) was dissolved in 10 cc. of methyl alcohol saturated with ammonia at 0°. After 2 days at room temperature, the solution was evaporated down and the residue dissolved in dilute bicarbonate solution. The substance obtained on acidifying with dilute hydrochloric acid was recrystallized from dioxane-ether. M.p., 181°.

$C_{22}H_{25}O_7N_3$. Calculated. C 59.6, H 5.7, N 9.5
443.4 Found. " 59.4, " 5.8, " 9.4

Carbobenzoxy-l-Glutaminyl-l-Tyrosineamide—2.1 gm. of carbobenzoxyglutamyltyrosine ethyl ester were esterified in the usual manner with methyl alcohol saturated with hydrogen chloride. The syrupy ester obtained on evaporation was treated with a methyl alcohol solution of dry ammonia. The diamide crystallized out on allowing the reaction mixture to stand at room temperature. Yield, 1.8 gm. M.p., about 240°.

$C_{22}H_{26}O_6N_4$. Calculated. C 59.7, H 5.9, N 12.7
442.5 Found. " 59.4, " 6.1, " 12.6

Carbobenzoxy-l-Glutamyl-l-Tyrosylglycine

Carbobenzoxy-l-Glutamyl-l-Tyrosine Hydrazide—4.7 gm. of the corresponding ethyl ester (1) were treated with 1.5 cc. of hydrazine hydrate in 5 cc. of absolute alcohol. Slight warming gave a clear solution and on standing overnight the hydrazide separated out. Yield, 4.5 gm. On recrystallization from absolute alcohol the substance melted at 194°.

$C_{22}H_{26}O_7N_4$. Calculated. C 57.6, H 5.7, N 12.2
458.5 Found. " 57.4, " 5.6, " 11.9

Carbobenzoxy-l-Glutamyl-l-Tyrosylglycine Ethyl Ester—2.3 gm. of the above hydrazide were suspended in 25 cc. of ice-cold water, dissolved by the addition of 6 cc. of concentrated hydrochloric acid, and converted to the azide by the addition of 0.5 gm. of sodium nitrite. The azide was extracted with ethyl acetate and the extract was washed repeatedly with ice-cold water. The dry azide solution was then added to a dry ether solution of glycine ethyl ester (from 5 gm. of the hydrochloride). After 24 hours the reaction mixture was washed with dilute hydrochloric acid and water. On evaporation of the ether-ethyl acetate layer, 1.7 gm. of the expected product were obtained. After recrystallization from dioxane-ether the melting point was 193–194°.

$C_{26}H_{31}O_9N_3$. Calculated. C 58.9, H 5.9, N 7.9
529.5 Found. " 58.6, " 5.9, " 8.0

Carbobenzoxy-l-Glutamyl-l-Tyrosylglycine—2.5 gm. of the above ester were suspended in absolute alcohol and treated with 14 cc. of N NaOH. The resulting solution was left at 20° for 1 hour, acidified to Congo red with N hydrochloric acid, and concentrated down. The syrup which separated crystallized on standing. Yield, 2.0 gm. The material was air-dried for analysis. A sample dried *in vacuo* at 100° for 6 hours over P_2O_5 melted at 182°.

$C_{24}H_{27}O_9N_3 \cdot H_2O$. Calculated. C 55.5, H 5.6, N 8.1, H_2O 3.5
519.5 Found. " 55.7, " 5.4, " 8.2, " 3.4

The enzymatic hydrolysate by papain of 250 mg. of this substance was concentrated to a small volume and acidified to Congo red. The resulting oil was extracted with ethyl acetate. The ethyl acetate layer was washed with water and then extracted with dilute bicarbonate solution. The bicarbonate extract was acidified to give a syrup which crystallized on standing in the ice chest. The dry material (86 mg.) melted at 118–120° and gave a mixed melting point of 119° with an authentic sample of carbobenzoxy-*l*-glutamic acid.

Carbobenzoxy-*l*-glutamic acid
$C_{13}H_{15}O_6N$. Calculated. C 55.5, H 5.4, N 5.0
281.2 Found. " 55.3, " 5.3, " 5.0

Carbobenzoxyglycyl-l-Glutamyl-l-Tyrosine

l-Glutamyl-l-Tyrosine Ethyl Ester—4.7 gm. of carbobenzoxy-*l*-glutamyl-*l*-tyrosine ethyl ester were hydrogenated in alcohol in the usual manner. Yield, 3.2 gm. The substance was recrystallized from hot alcohol. M.p., 144°.

$C_{16}H_{22}O_6N_2$. Calculated. C 56.7, H 6.6, N 8.3
338.4 Found. " 56.4, " 6.4, " 8.2

Carbobenzoxyglycyl-l-Glutamyl-l-Tyrosine Diethyl Ester—3.1 gm. of glutamyltyrosine ester were esterified twice in the usual manner with ethanol-HCl. The syrupy diethyl ester hydrochloride was converted to the free ester with potassium carbonate. To the ethyl acetate solution of the free ester, 2.3 gm. of carbobenzoxyglycyl chloride were added in two portions with shaking. The reaction mixture was then shaken with a dilute bicarbonate solution. The ethyl acetate layer was then washed successively with water, dilute hydrochloric acid, and water. Crystallization occurred when the dried ethyl acetate solution was concentrated down. Yield, 4.3 gm. The substance was recrystallized from ethyl acetate. M.p., 169°.

$C_{28}H_{35}O_9N_3$.	Calculated.	C 60.3, H 6.3, N 7.5
557.6	Found.	" 60.3, " 6.4, " 7.6

Carbobenzoxyglycyl-l-Glutamyl-l-Tyrosine—2.8 gm. of the diethyl ester were suspended in 25 cc. of ethanol and with cooling there were slowly added 15 cc. of N NaOH. After 1 hour, 16 cc. of N HCl were added (Congo red acidity). The material which crystallized out on standing was filtered off and air-dried. Yield, 2.1 gm. A sample dried *in vacuo* at 100° for 5 hours over P_2O_5 melted at 173°.

$C_{24}H_{27}O_9N_3 \cdot H_2O$.	Calculated.	C 55.5, H 5.6, N 8.1, H_2O 3.5
519.5	Found.	" 55.3, " 5.6, " 8.0, " 3.7

During the enzymatic hydrolysis of 130 mg. of this substance by pepsin a crystalline precipitate separated out. It was filtered off and dried (20 mg.). Calculated for tyrosine, 7.7 per cent of NH_2-N; found, 7.8 per cent of NH_2-N.

Glycyl-l-Glutamyl-l-Tyrosine—1 gm. of the carbobenzoxy compound was hydrogenated in the usual manner. The tripeptide crystallized out on evaporation of the filtrate. Yield, 0.6 gm.

$C_{16}H_{21}O_7N_3 \cdot 2\frac{1}{2}H_2O$.	Calculated.	C 46.6, H 6.4, N 10.2, H_2O 10.9
412.3	Found.	" 46.8, " 6.4, " 10.2, " 10.9

Carbobenzoxy-l-Glutaminyl-l-Phenylalanine

Carbobenzoxy-l-Glutaminyl-l-Phenylalanine Ethyl Ester—2.15 gm. of carbobenzoxyglutamylphenylalanine ethyl ester were suspended in 35 cc. of dry chloroform. 1.1 gm. of PCl_5 were added, followed by shaking for 5 minutes at 0° and subsequent removal of the HCl by evacuation for 10 minutes. The solution was then added with cooling to an ethereal solution of ammonia. After the solution had stood at room temperature for 1 hour, the precipitate was filtered off, washed thoroughly with cold water, and dried. Yield, 1.5 gm. M.p., 138°.

$C_{24}H_{29}O_6N_3$.	Calculated.	C 63.3, H 6.4, N 9.2
455.5	Found.	" 63.4, " 6.4, " 9.1

Carbobenzoxy-l-Glutaminyl-l-Phenylalanine—1.15 gm. of the above ester were dissolved in 60 cc. of alcohol and 2.5 cc. of N NaOH were added. The solution was acidified to Congo red after standing 1 hour at room temperature. The substance crystallized out upon concentration under reduced pressure. Yield, 0.9 gm. M.p., 180°.

$C_{22}H_{25}O_6N_3$.	Calculated.	C 61.8, H 5.9, N 9.8
427.4	Found.	" 61.9, " 6.1, " 9.7

Carbobenzoxy-l-Phenylalanyl-l-Glutamic Acid

Carbobenzoxy-l-Phenylalanyl-l-Glutamic Acid Diethyl Ester—3.5 gm. of carbobenzoxy-*l*-phenylalanyl chloride were added to a dry ether solution of glutamic acid diethyl ester (prepared from 6 gm. of the hydrochloride). The mixture was allowed to stand at room temperature for 1 hour and washed with dilute hydrochloric acid, water, and bicarbonate solution. The dried ethereal solution was concentrated, yielding a crystalline product. Yield, 3.7 gm. M.p., 115°.

$C_{26}H_{32}O_7N_2$.	Calculated.	C 64.4, H 6.7, N 5.8
484.5	Found.	" 64.5, " 6.7, " 5.8

Carbobenzoxy-l-Phenylalanyl-l-Glutamic Acid—2.4 gm. of the above ester were shaken with a mixture of 10 cc. of N NaOH and 10 cc. of alcohol. After 1 hour the solution was acidified to Congo red with N hydrochloric acid and concentrated under reduced pressure. The resulting crystals were filtered off and washed with cold water. Yield, 1.5 gm. M.p., 180°.

$C_{22}H_{24}O_7N_2$.	Calculated.	C 61.7, H 5.6, N 6.5
428.4	Found.	" 61.5, " 5.7, " 6.5

Carbobenzoxy-l-Glutamyl-l-Glutamic Acid—This compound was prepared as described in (8).

Carbobenzoxy-l-Tyrosyl-l-Tyrosine—This compound was prepared as described in (8).

l-Glutamyl-l-Tyrosine—This compound was prepared as described in (8).

Carbobenzoxy-l-Glutamylglycine—This compound was prepared as described in (9).

Carbobenzoxyglycyl-l-Tyrosine—This compound was prepared as described in (7).

Enzymatic Studies

The crystalline pepsin was prepared according to the directions of Philpot (10). Unless otherwise stated, twice crystallized preparations were employed. Solutions of the enzyme were made up in acetate buffer of pH 4.0.

The concentration of the synthetic substrates was 0.05 mM per cc. in all cases. The solutions were buffered by 0.1 M acetate buffers. The temperature in all cases was 40°. The extent of hydrolysis was followed by the determination of the amino nitrogen liberated in the Van Slyke microvolumetric apparatus. Enzyme blanks and controls to test the lability of the substrates in the absence of pepsin were performed throughout these investigations.

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THE SPECIFICITY OF TRYPSIN

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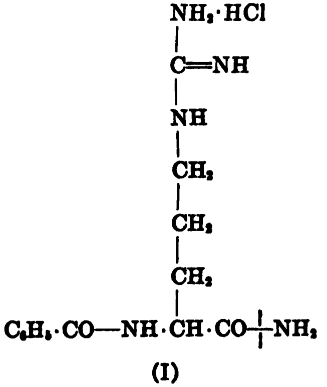
(Received for publication, December 13, 1938)

The isolation of two proteinases in crystalline form from extracts of beef pancreas has been described by Kunitz and Northrop (1). These enzymes have been named trypsin and chymotrypsin. Previous publications (2) from this laboratory have reported the finding of a series of synthetic peptide derivatives which were readily hydrolyzed by crystalline chymotrypsin. In this communication a synthetic substrate for crystalline trypsin is described.

α-Benzoyl-*l*-arginineamide hydrochloride (I) is hydrolyzed extremely rapidly by crystalline trypsin (recrystallized three times) to yield benzoyl-*l*-arginine and ammonia (*cf.* Table I). This hydrolysis proceeds optimally at about pH 7.8 (Fig. 1).



$$\begin{array}{l} \qquad\qquad\qquad\quad NH_2\cdot HCl \\ \qquad\qquad\qquad\quad | \\ \qquad\qquad\qquad\quad C{=}NH \\ \qquad\qquad\qquad\quad | \\ \qquad\qquad\qquad\quad NH \\ \qquad\qquad\qquad\quad | \\ \qquad\qquad\qquad\quad CH_2 \\ \qquad\qquad\qquad\quad | \\ \qquad\qquad\qquad\quad CH_2 \\ \qquad\qquad\qquad\quad | \\ \qquad\qquad\qquad\quad CH_2 \\ \qquad\qquad\qquad\quad | \\ C_6H_5\cdot CO{-}NH\cdot CH\cdot CO \,\vdots\, NH_2 \end{array}$$

(I)

Thus far, this compound is the only peptide-like derivative that has been found to act as a substrate for crystalline trypsin. Even the closely related α-toluenesulfonyl-*l*-arginineamide hydrochloride is not hydrolyzed. Experiments with a series of acylamino acid amides containing histidine, lysine, glutamic acid, tyrosine, and glycine gave negative results. Furthermore, trypsin is unable to

TABLE I

Action of Proteinases on Benzoyl-l-Arginineamide

Enzyme	pH	Time	Hydrolysis	Isolation of products
		hrs.	*per cent*	
Trypsin, 0.25 mg. protein N per cc.	7.8	1	50	Benzoyl-*l*-arginine
		2	64	
		3	80	
		20	95	
Chymotrypsin, 0.7 mg. protein N per cc.	7.8	2	1	
		19.5	3	
		43	4	
Papain-HCN*	5.0	2	76	
		6	96	
		24	99	
Bromelin-HCN*	5.0	6	45	
		24	77	

* The experiments with papain and bromelin were set up exactly as described in the experimental part of (3).

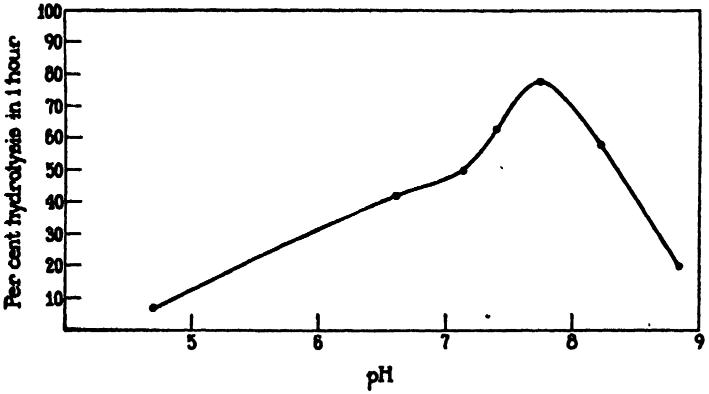


FIG. 1. pH dependence of hydrolysis of benzoyl-*l*-arginineamide by crystalline trypsin. Enzyme concentration 0.4 mg. of trypsin N per cc.

split the substrates of chymotrypsin (benzoyl-*l*-tyrosylglycineamide) or heterotrypsin (α-hippuryllysineamide) (*cf.* Table II).

The differences in chemical specificity exhibited by the three pancreatic enzymes trypsin, chymotrypsin, and heterotrypsin permit of a determination of their respective activities in crude pancreatic extracts. Such a study is at present in progress in this laboratory. The availability of the synthetic substrates also facilitates the study of the relationship of the pancreatic proteinases to enzymes obtained from other biological sources and named "trypsins" because of their optimal action on proteins in an alkaline milieu.

TABLE II

*Behavior of Synthetic Substrates in Presence of Trypsin**

Substrate	Time	Hydrolysis
	hrs.	*per cent*
Toluenesulfonyl-*l*-arginineamide	24	−1
Benzoyl-*l*-histidineamide	42	3
Benzoyl-*l*-lysineamide	42	2
Carbobenzoxy-*l*-isoglutamine	48	3
Benzoyl-*l*-tyrosineamide	48	0
Hippurylamide	48	1
Benzoyl-*l*-tyrosylglycineamide	24	0
α-Hippuryl-*l*-lysineamide	24	4

* pH 7.8; 0.4 mg. of trypsin N per cc.

The behavior of benzoylarginineamide when subjected to the action of other proteinases is of some interest (*cf.* Table I). The resistance of this compound to chymotrypsin emphasizes the difference in chemical specificity of trypsin and chymotrypsin. Furthermore, the rapid hydrolysis of this substrate by the intracellular enzymes papain and bromelin is noteworthy. The use of benzoylarginineamide for the study of the activation of papain has been described elsewhere (4).

The closer study of the specificity of crystalline trypsin, with suitably substituted peptides of arginine and other amino acids, is contemplated.

EXPERIMENTAL

α-Benzoyl-l-Arginineamide Hydrochloride[1]

Benzoyl-l-Arginine—To a solution of 21.2 gm. of arginine monohydrochloride in 120 cc. of H_2O there were added in several portions 12.8 cc. of benzoyl chloride and a solution of 17.2 gm. of sodium carbonate in 170 cc. of water. The reaction mixture was stirred vigorously and the temperature kept at about 20°. The addition of the reagents required 2½ hours, and after another 1½ hours of stirring the reaction mixture was acidified to Congo red and the slight precipitate (dibenzoylarginine) filtered off. The filtrate was extracted with ether and neutralized to about pH 8 with ammonia. Upon concentration under diminished pressure, crystallization occurred. Yield, 20 gm. The material was recrystallized from hot water.

$C_{13}H_{18}O_3N_4$. Calculated. C 56.0, H 6.5, N 20.2
278.3 Found. " 55.8, " 6.4, " 19.9

Benzoyl-l-Arginineamide Hydrochloride—7.6 gm. of the above compound were esterified in the usual manner (with cooling) in 350 cc. of absolute methanol. After the reaction mixture had stood in the ice box overnight, the solution was concentrated at 30°. The residue was dissolved in a small volume of methanol and the syrupy ester precipitated by means of dry ether. This material was reesterified and the resulting syrup was dissolved in 70 cc. of methanol previously saturated with dry ammonia at 0°. After it had stood 2 days at room temperature, the solution was concentrated and the resulting syrup taken up in hot water. The material which crystallized on cooling sintered at 120–123°, lost water of crystallization at 135–140°, and decomposed above 260°. Yield, 3.7 gm.

$C_{13}H_{20}O_2N_5Cl \cdot H_2O$. Calculated. C 47.1, H 6.6, N 21.1
331.8 Found. " 47.0, " 6.6, " 21.0

The enzymatic hydrolysate by trypsin of 498 mg. of this compound was filtered, concentrated to a small volume, and placed in the ice box. The resulting crystals were collected, washed with cold water, dried (288 mg.), and recrystallized from hot water.

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[1] This compound has already been synthesized by Dirr and Späth (5). In view of its value as a substrate for trypsin, a fuller description of the synthesis is presented.

Benzoyl-*l*-arginine
$C_{13}H_{18}O_3N_4$. Calculated. C 56.0, H 6.5, N 20.2
278.3 Found. " 55.9, " 6.5, " 19.8

During the enzymatic hydrolysis the liberation of ammonia was also observed.

α-Toluenesulfonyl-l-Arginineamide

α-Toluenesulfonyl-l-Arginine—This compound has already been described as an oil by McChesney and Swann (6). It was obtained in crystalline form by the following procedure.

21 gm. of arginine monohydrochloride were dissolved in 115 cc. of 2 N NaOH and stirred with a solution of *p*-toluenesulfochloride in 400 cc. of ether. After about 25 minutes the formation of crystals began. 3 hours later the mixture was acidified by addition of acetic acid, and the crystals of toluenesulfonylarginine were filtered after several hours. Yield, 36 gm. On recrystallization from hot water, the substance melted at 256–257° (decomposition).

$C_{13}H_{20}O_4N_4S \cdot 3H_2O$. Calculated. C 40.8, H 6.8, N 14.7, H_2O 14.1
382.3 Found. " 40.7, " 6.7, " 14.6, " 14.1

α-Toluenesulfonyl-l-Arginineamide Hydrochloride—7 gm. of dry toluenesulfonylarginine were treated at 0° with 500 cc. of absolute methanol saturated with dry HCl. After 18 hours the methanol was evaporated off and the resulting syrup crystallized by means of ether. This material was treated with 60 cc. of absolute methanol previously saturated with dry ammonia at 0°. After 48 hours, the evaporated solution yielded crystals which were transferred to the filter by means of ether. Yield, 6.2 gm.

$C_{13}H_{22}O_3N_5SCl \cdot H_2O$. Calculated. C 40.9, H 6.3, N 18.3, H_2O 4.7
381.8 Found. " 41.0, " 6.2, " 18.4, " 4.8

Benzoyl-l-Histidineamide—1 gm. of monobenzoylhistidine methyl ester (7) was dissolved in 10 cc. of methanol previously saturated with dry ammonia. The amide crystallized on standing at room temperature for 18 hours. After recrystallization from methanol the substance melted at 234°.

$C_{13}H_{14}O_2N_4$.	Calculated.	C 60.4, H 5.3, N 21.6
258.2	Found.	" 60.4, " 5.4, " 21.6

Benzoyl-l-Lysineamide—This substance was prepared as described in (8).

α-Hippuryl-l-Lysineamide—This substance was prepared as described in (8).

Carbobenzoxy-l-Isoglutamine—This substance was prepared as described in (9).

Benzoyl-l-Tyrosineamide—This substance was prepared as described in (2).

Benzoyl-l-Tyrosylglycineamide—This substance was prepared as described in (2).

Enzymatic Studies

Trypsin, recrystallized three times, and chymotrypsin (prepared as described in (1)) were employed. The concentration of the synthetic substrates was 0.05 mM per cc. in all cases. The solutions were buffered at pH 7.8 by means of M/15 phosphate buffers and at pH 5 by means of 0.04 M citrate buffers. The temperature was 40° in all experiments. The extent of hydrolysis was followed by microtitration of the liberated carboxyl groups according to the method of Grassmann and Heyde (10). Enzyme blanks and controls to test the lability of the substrates in the absence of the enzyme were performed.

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A NEW PRINCIPLE FOR THE DETERMINATION OF AMINO ACIDS, AND ITS APPLICATION TO COLLAGEN AND GELATIN

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The fundamental task of protein chemistry is the establishment of the composition of the various proteins in terms of the constituent amino acids in the same manner as the composition of simpler molecules has been ascertained in terms of their constituent atoms. We have attempted, therefore, to develop a general principle that would permit the determination of amino acids to be made with a precision approaching that attained in the elementary analysis of simpler molecules. The present communication contains a description of this new principle, and of its application to the determination of glycine, *l*-alanine, *l*-leucine, and *l*-proline in solutions of known content. In addition, determinations of *l*-proline and glycine in hydrolysates of cattle tendon collagen and of commercial gelatin preparations are described.

Analytical Procedure

All the methods hitherto described for the determination of amino acids have one feature in common. In each instance an attempt is made to isolate as much as possible of a given amino acid—either as such or as a salt, a derivative, or another transformation product. With respect to the precipitation of amino acids as salts, all efforts have been directed towards the discovery of reagents permitting the quantitative removal of an amino acid from protein hydrolysates. The well-nigh insurmountable difficulties attending the development of a reagent which will selectively and quantitatively remove any amino acid from so complex a mixture are evidenced by the fact that no reagent fulfilling these requirements has as yet been found. It has frequently been suggested that the accuracy of determinations involving salt precipitations could be improved by incorporating in the final result a correction which allows for the solubility of the salt in the mother liquor. The fact has been neglected, however, that the solubility of an amino acid salt in its mother liquor is a rather complex function of the composition of this mother liquor. Without any knowledge of the nature of this function, it is impossible to ascertain how much of an amino acid salt has, under given conditions, escaped precipitation. In consequence, there is a varying element of doubt attached to all the figures for the amino acid content of a protein which have been obtained with the aid of methods employing salt precipitations.

The experiments reported in the present paper were undertaken to study the solubility behavior of amino acid salts. These studies resulted in a method for the determination of amino acids which foregoes any attempt at a complete precipitation of an amino acid as a salt. On the contrary, in the new method only those amino acid salts that possess a measurable solubility can be employed (1).

This method is founded upon our experimental observation that the amount of an amino acid salt precipitated from a solution is, at equilibrium, a function of the concentration of its ions in solution. The general case under investigation is that in which an amino acid A combines with an acidic reagent RH to form the binary salt AH·R. It was found that the quantity of the salt AH·R, which crystallizes from a solution containing the ions AH^+ and R^-, is such that at the completion of the precipitation the product of the concentrations of the ions AH^+ and R^- remaining in solution has assumed a value that is a constant for a given solution at a given temperature.

$$[AH^+] \times [R^-] = \text{constant} \qquad (1)$$

In order to discuss the applicability of Equation 1 to the determination of an amino acid, the following example may be considered. To a solution containing Y moles of an amino acid in a volume of v ml. are added r' moles of reagent. After equilibrium has been attained, the precipitated amino acid salt is filtered, weighed, analyzed, and thus found to contain x_1 moles of the reagent. The difference $(r' - x_1)$ is the quantity of reagent remaining in solution. This quantity, when reduced to a 1 liter basis, gives the concentration of the reagent remaining in solution. The concentration of A remaining in solution may also be found from the weight and composition of the precipitate, for the precipitate contains equimolar amounts x_1 of reagent and y_1 of amino acid. Therefore $Y - y_1$ is the amount of A remaining in solution. This quantity must also be reduced to a 1 liter basis in order to obtain the concentration of the amino acid remaining in solution.

If one now employs the concentrations of amino acid A and reagent RH remaining in solution, instead of the concentrations of the respective ions AH^+ and R^-, Equation 1 may be written in the following form.[1]

$$\frac{1000(r' - x_1)}{v} \times \frac{1000(Y - y_1)}{v} = C \qquad (1, a)$$

The constant, C, and Y are the only unknowns in Equation 1, *a*. In order to eliminate the constant, it is necessary only to perform a second experiment in which an identical sample of amino acid solution is employed, but a larger amount of reagent r''. A larger amount of precipitate is formed in this experiment, but it again contains equivalent molar quantities x_2 of reagent and y_2 of amino acid. This second experiment can be represented by Equation 1, *b*.

$$\frac{1000(r'' - x_2)}{v} \times \frac{1000(Y - y_2)}{v} = C \qquad (1, b)$$

A combination of the Equations 1, *a* and 1, *b* gives Equation 2.

$$(r' - x_1)\,(Y - y_1) = (r'' - x_2)\,(Y - y_2) \qquad (2)$$

From this equation the value for Y, the amino acid content of the sample originally taken, may readily be calculated.

Fig. 1 illustrates an experiment in which r' and r'' moles of a reagent are added to two equal samples of a solution, each containing Y moles of amino acid. The moles of reagent added are plotted against the moles of amino acid present. It is apparent that when the precipitation has ceased, x' moles of reagent and y' moles of amino acid remain in the solution to which r' moles of reagent were added. Similarly, x'' moles of reagent and y'' moles of amino acid remain in the solution to which r'' moles of reagent were added. Thus we find that the course of the precipitation with increasing amounts of reagent follows a hyperbolic curve passing through the points C' and C''.

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[1] It will be noticed that in substituting the concentrations of A and RH for the concentrations of AH^+ and R^-, the activity coefficients of these ions are neglected. The assumption is made that the activities, whatever they may actually be, remain nearly constant over the concentration range covered by the analytical precipitations. The admissibility of this assumption is supported by the experimental data reported in Table I.

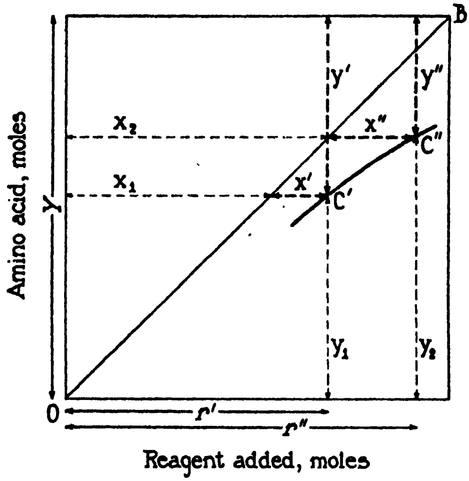

FIG. 1. Determination of amino acids by means of the solubility product. The symbols have the values described in the text.

From Fig. 1 it follows that Equation 2 may also be written

$$x' \times y' = x'' \times y'' = C \qquad (2, a)$$

This constant, reduced to a 1 liter basis, is usually referred to as the solubility product of the salt. Since the new analytical method is based upon the law of the constancy of the solubility product, it may, for the sake of brevity, be designated the solubility method.

In Table I are reported the experimental data from a number of determinations—made with the solubility method—of amino acids in solutions of known content.

TABLE I*

Amino Acid Analyses by Solubility Method

Amino acid	Amino acid content employed	Reagent employed	Amino acid pptd.	Reagent left in solution	Amino acid content found	Solubility product K
	mole	*mole*	*mole*	*mole*	*mole*	
l-Proline	0.00420 in 205 cc. 0.075 N HCl + 135 cc. methanol	0.00427†	0.00323	0.00104		8.72×10^{-6}
		0.00504	0.00353	0.00151	0.00419	8.80×10^{-6}
l-Alanine	0.00299 in 15 cc. N HCl	0.00188‡	0.00157	0.00031		1.96×10^{-3}
		0.00282	0.00223	0.00059	0.00300	1.99×10^{-3}
"	0.00300 in 15 cc. N HCl	0.00187‡	0.00151	0.00036		2.39×10^{-3}
		0.00281	0.00217	0.00064	0.00302	2.36×10^{-3}
l-Proline	0.0150 in 75 cc. N HCl	0.00936‡	0.00758	0.00178		2.34×10^{-3}
		0.01405	0.01084	0.00321	0.0149	2.37×10^{-3}
l-Leucine	" "	0.00936‡	0.00885	0.00051		5.55×10^{-4}
		0.01405	0.01274	0.00131	0.0152	5.24×10^{-4}
Glycine	" "	0.00585‡	0.00365	0.00220		4.42×10^{-3}
		0.01405	0.00954	0.00451	0.0152	4.36×10^{-3}
l-Proline + glycine	0.0150 + 0.00500 in 75 cc. N HCl	0.00936‡	0.00771	0.00165		2.14×10^{-3}§
		0.01405	0.01111	0.00294	0.0154	2.03×10^{-3}§
l-Leucine + *l*-proline	0.00790 + 0.00381 in 75 cc. 0.91 N HCl	0.00468‡	0.00394	0.00074		5.18×10^{-4}‖
		0.00707	0.00576	0.00130	0.00817	4.92×10^{-4}‖

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* We should like to state that we attach no great significance to the absolute values of the solubility products reported in the last column. The complex radicals used as precipitating agents undergo a slow decomposition. In addition, the various determinations were made under slightly different experimental conditions, exerting varying influences upon the solubility products. From the analytical point of view it is important, however, that the two simultaneous precipitations of each determination were performed under identical experimental conditions and consequently resulted in identical solubility products. For example, the two alanine determinations in Table I differ from each other in the absolute values of their solubility products. However, the solubility product is a constant for the two simultaneous precipitations of each determination. Moreover, the alanine values obtained in the two independent determinations are in good agreement with each other and with the amount of alanine actually present.

† Ammonium rhodanilate.

‡ Sodium dioxpyridate.

§ For proline.

‖ For leucine.

The high degree of accuracy achieved in the determinations reported in Table I is indeed gratifying and may, in part, be a consequence of the paucity and simplicity of the manipulations required in each determination. In applying the solubility method to protein hydrolysates, it is essential to preserve this simplicity in so far as possible. To this end it is advisable to perform each precipitation directly upon a fresh sample of hydrolysate, all manipulative procedures which might entail losses being avoided.

The solubility method should have a wide application in the determination of amino acids, for there are many reagents which form crystalline salts with amino acids; some of these reagents will be described in succeeding papers of this series. At present it may be pointed out that the solubility method is by no means restricted to the determination of amino acids, but may be applied also to the determination of a variety of constituents of complex mixtures.

One of the advantages of the solubility method is the relative ease with which the accuracy of the results obtained by it can be checked. There are several checks that can be applied. In the first place, when one is dealing with solutions of unknown amino acid content, it is advisable to perform three or four precipitations (rather than only two) with equal samples of the amino acid solution but increasing amounts of reagent. From a series of three precipitations, three values for the amino acid content of a solution may be calculated; while from a series of four precipitations, six values may be calculated. None of the values should be accepted unless all of them are in good agreement. In the second place, the determination may be repeated with a different concentration of the amino acid. The results of the determinations performed at different concentrations should check one another. In the third place, a known quantity of the amino acid under investigation may be added to the analytical solution before the precipitations are performed. The result of the determination, reduced by the amount of amino acid added, should closely approximate the result obtained when no amino acid is added. The description of the application of these checks to the determination of *l*-proline in protein hydrolysates will be found in the experimental section of this paper.

In the application of the solubility method two types of reagents are available. One type comprises those reagents which are specific for a single amino acid and do not precipitate other amino acids under ordinary conditions. Rhodanilic acid, when used as its ammonium salt, seems to represent this type of reagent, inasmuch as it precipitates only proline (2). A second, and more general type of reagent, forms salts with a variety of amino acids. These salts, however, frequently exhibit differences in the values of their solubility products (Table I). Since the relative concentrations of amino acids in the hydrolysates of different proteins vary within wide limits, the same reagent may be used for the determination of one amino acid in one hydrolysate and of another amino acid in another hydrolysate. In order to illustrate this procedure in an artificial mixture, there are described in this paper determinations, made with dioxpyridic acid (3), of *l*-leucine in a mixture of *l*-leucine and *l*-proline, and of *l*-proline in a mixture of *l*-proline and glycine.

When a reagent of the second type is applied to the determination of any component of a mixture of amino acids of unknown content, either or both of the following procedures may be employed to insure the precipitation of a single amino acid salt. The mixture may be diluted to a point at which the solubility product of only one component of the mixture with the reagent is surpassed. At such a dilution only a single amino acid salt is precipitated. Sometimes, however, the various amino acids are present in such relative amounts that there can be found no dilution at which only one amino acid is precipitated by a given reagent. This difficulty may be overcome by the addition of a known quantity of the amino acid under determination. These procedures will be exemplified in forthcoming papers on the composition of proteins.

Protein hydrolysates sometimes contain several stereoisomeric forms of an amino acid. If both the *l* and the *d* forms of an amino acid are present, they will behave, in the course of a determination by the solubility method, as two amino acids, the salts of which, with a symmetrical reagent, possess identical solubility products. If there is a preponderant quantity of one of the stereoisomers, the determination may be performed at such a dilution that only the predominating isomer is precipitated. If, on the other hand, both the *l* and the racemic form of an amino acid are present in solution, they may behave as two different amino acids, the salts of which possess different solubility products.

The accuracy of the solubility method so far surpasses that of the methods hitherto available that it seems worth while to apply it to a reinvestigation of the amino acid content of many proteins. Thus we intend to subject the periodicity theory of protein structure to a series of rigorous checks.[3]

Determinations of l-Proline and Glycine in Gelatin and Collagen

In order to test the applicability of the solubility method to protein hydrolysates, *l*-proline was determined in hydrolysates of gelatin and of collagen of cattle Achilles tendon. For a comparison the glycine content of these proteins was reinvestigated. Glycine was precipitated by means of potassium trioxalatochromiate, according to the procedure recently described (7). The results of these glycine determinations are reported in Table II, while the experimental details will be found in the experimental section of this paper.

The agreement among these values is rather striking. It indicates that the glycine determination, by means of potassium trioxalatochromiate, is sufficiently precise to permit a comparative study of the collagens of different tissues and of various species. In this respect it seems of interest that the glycine content of the collagen and the gelatins investigated is the same.

The average glycine content, 26.5 per cent, found for several gelatins in these experiments is only slightly higher than the 25.5 per cent found by Dakin (8).

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[3] Doubts as to the validity of the periodicity theory have recently been expressed by Astbury (4). After discussing the stoichiometry of the keratin molecule on the basis of the available constituent analyses of sheep wool, he emphasizes that frequencies such as eight and nine cannot coexist within the same protein molecule. As a matter of fact, the coexistence of these frequencies has never been claimed. Moreover, we feel that constituent analyses of protein mixtures of varying composition, such as the varieties of sheep wool, should not be employed as a basis for the calculation of amino acid ratios within the keratin molecule. Still less acceptable is the calculation, by Astbury, of the leucine ratio within the keratin molecule on the basis of a leucine determination performed with sheep wool in 1907 by Abderhalden and Voitinovici (5) by means of the ester method; *i.e.*, by a method known to give incomplete yields of a mixture of leucine, isoleucine, and valine (6).

With the figure of 26.5 per cent glycine, one may easily calculate that 100 gm. of water and ash-free gelatin or collagen would yield 0.353 gm. equivalent of glycine. The average weight of all the known amino acid residues in gelatin is about 89.2. This value is, in all probability, too low, inasmuch as the amino acid residues yet to be determined in gelatin may possess a higher average residue weight. We suggest an average residue weight of about 125 as a fair estimate for these undetermined residues. If these residues are taken into account, the corrected average residue weight for the total number of amino acid residues in gelatin would be 93.7. 100 gm. of gelatin therefore should yield on complete hydrolysis 1.067 gm. equivalents of the average amino acid. If one out of every three amino acids in gelatin were glycine, then 100 gm. of the protein should yield 0.356 gm. equivalent of glycine or 26.7 per cent.[3] The values found are in excellent agreement with this calculated figure. It appears therefore that one out of every three amino acid residues in gelatin and in collagen is indeed glycine, as has already been suggested by Astbury (9) on the basis of Dakin's glycine estimation (8).

TABLE II

Glycine Content of Several Protein Hydrolysates

Protein	Amount of protein employed	Amount of glycine added	Total N of ppt.	NH_3-N of ppt.	Glycine found	Glycine in protein
	gm.	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
Pig skin gelatin	1.81		82.6	1.8	471	26.0
	0.905	230	81.5	1.4	466	26.1
Coignet Gold Label gelatin	1.72		81.2	1.3	465	27.0
	0.860	230	80.0	0.9	461	26.9
Cattle Achilles tendon collagen	1.56		72.1	1.9	409	26.2
	0.780	230	76.5	1.3	438	26.7

By means of the solubility method, proline was determined as rhodanilate by precipitation with ammonium rhodanilate. The method of procedure will be found in the experimental section.

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[3] If the average residue weight of the undetermined amino acids in gelatin were 130 instead of 125, then 100 gm. of gelatin should yield 0.355 gm. equivalent of glycine or 26.6 per cent.

The values obtained are reported in Table III. The average of the proline estimations in two commercial gelatins and in collagen of cattle Achilles tendon is 17.5. The single determinations do not deviate from the average by more than 2.5 per cent of the final value.

In an earlier paper from this laboratory, the value 19.7 per cent was reported for the proline content of gelatin. Our earlier value was obtained from experiments in which we endeavored to precipitate proline as completely as possible and to apply an empirical correction. As is pointed out in the present communication, values obtained by a "maximum precipitation" method can never be thoroughly accurate.

TABLE III

Proline Content of Several Protein Hydrolysates

Protein	Amount of protein employed	Total volume	Proline added	Proline found in protein	No. of precipitations
	gm.	*cc.*	*gm.*	*per cent*	
Pig skin gelatin	2.53	330		17.5	3
	2.48	450		17.6	3
	1.27	330	0.249	17.7	3
Coignet Gold Label gelatin	2.59	330		17.0	3
	2.59	330		17.6*	3
	1.21	330	0.250	17.4	2
Cattle Achilles tendon collagen	2.33	330		17.1	3
	1.09	330	0.251	17.8	3

* This determination was performed by Dr. H. R. Ing in this laboratory.

On the other hand, there is as yet no proof that the value 17.5 per cent, obtained for *l*-proline by the solubility method, represents the total proline content of the original gelatin and collagen. It seems possible that a part of the proline is racemized during the hydrolysis of the protein with boiling hydrochloric acid. The extent of racemization during hydrolysis is at present being thoroughly investigated in this laboratory. In the event that this investigation should reveal only an inconsequential racemization, thus making the total proline content of gelatin and collagen about 17.5 per cent, one may easily calculate that the numerical ratios of proline to glycine to all amino acid residues are approximately 3:7:21. It is apparent that the proline residues cannot be distributed through the peptide chains of gelatin and collagen in a manner so that they recur regularly with a frequency of seven. It may become necessary, therefore, to assign a dual frequency to the distribution of proline in gelatin and collagen. However, we should like to reserve the discussion of the stoichiometry of gelatin and collagen pending the results of further investigations now in progress.

EXPERIMENTAL

General Remarks

When the solubility method is employed, the following precautions must be observed. The precipitating reagent used must be analytically pure. The recovered amino acid salt also should possess a high degree of purity, and its composition should be rigorously checked in every instance. The final concentration of the ions participating in the solubility product must be appreciable in comparison with the experimental errors involved in the determination of these concentrations. Suitable experimental conditions fulfilling these requirements must be found for each type of determination.

Finally, it should be emphasized that care must be taken to insure the establishment of a true equilibrium.

Sodium Dioxpyridate—This compound was prepared as described earlier (3). It was then dissolved in a minimum of water at room temperature, filtered, and salted-out by the addition of sodium acetate. After filtration it was suspended in about half its weight of ice-cold water, kept for 1 hour at 0°, and then sucked and dried on a porous plate to constant weight, first at 0° and then at room temperature. The salt was stored in the refrigerator.

Ammonium Rhodanilate—The salt was prepared as described previously (2). The product was then dissolved in cold methyl alcohol and, after addition of charcoal, filtered through a fluted filter. Ice water was added in several portions to the cooled filtrate. After filtration and washing with ice water, the recrystallization was repeated. The reagent was dried, protected from direct light on a porous plate, and stored in the refrigerator.

Determination of Glycine, l-Alanine, l-Leucine, and l-Proline As Dioxpyridates—As illustrative of the general case, the precipitation of *l*-proline with sodium dioxpyridate may be described in detail. 5.744 gm. of *l*-proline were dissolved in N HCl to a volume of 250 cc. To two 75 cc. portions of this solution were added 4.000 and 6.000 gm. of sodium dioxpyridate. The samples were first kept, in tightly stoppered flasks, at room temperature (22°) for 2 hours with shaking and then for 2 days at exactly 0° with frequent shaking. The precipitates were collected at 0° on weighed crucibles with sintered glass filter plates. The crucibles were dried in air at room temperature for 24 hours and then over $CaCl_2$ and NaOH to constant weight.

The data of this and other determinations will be found in Table I. There is also included an estimation of proline in presence of glycine and of leucine in presence of proline. In both determinations the purity of the precipitates was checked by amino nitrogen determinations. In the determination of proline the precipitate contained no amino nitrogen.

Determination of Proline As Rhodanilate—2.357 gm. of *l*-proline were dissolved in 75 cc. of N HCl and made up to a volume of 1 liter with water. The solution was cooled to 0°. Two cooled 205 cc. samples, each containing 483 mg. of proline, were then added to cooled solutions of 2.200 and 2.600 gm. samples of ammonium rhodanilate, each dissolved in 135 cc. of methanol. The mixtures were kept at exactly 0° for 16 hours, shaken at the same temperature for 4 hours, and again kept at 0° for 20 hours. The precipitates formed were collected at 0° on weighed crucibles with a sintered glass filter plate, each washed rapidly with 30 cc. of ice-cold water, and dried first in air and then over $CaCl_2$ to constant weight. The data from this experiment are found in Table I.

Preparation of Protein Hydrolysates—Protein hydrolysates prepared for constituent analysis by acid hydrolysis in the established manner cannot be employed for determination with the solubility method. The dark, colloidal decomposition products contained in such hydrolysates may inhibit the formation of precipitates in some cases, and in others the precipitates are highly contaminated. It is essential, therefore, to remove the decomposition products from the hydrolysate before attempting any analysis by the solubility method. It has been found that this may easily be accomplished by adding to the hydrolysate some pure $Cu(OH)_2$ and reprecipitating the copper with H_2S. The procedure has been successfully applied to hydrolysates of several proteins. The loss of amino acids, as measured by the determination of amino nitrogen, did not exceed 1.5 per cent and was, in most cases, negligible. The solutions resulting from this treatment have only a brownish yellow color and are optically clear.

Preparation of Collagen of Cattle Achilles Tendon—Cattle Achilles tendon was obtained in a fresh state from the slaughterhouse. The portion used was a strip extending 4 to 6 inches up the leg from the bifurcation at the hoof. Adhering fat and meat were removed, and the material was hashed by freezing with dry ice and passing through a grain grinder with large quantities of dry ice. In this manner the material remained frozen throughout the grinding process. The collagen was extracted at 0° for 2 weeks, with 10 per cent saline that was changed daily. The collagen was then successively extracted at 0° with M/15 Na_2HPO_4 for 3 days, with water until the extract gave a negative chloride test, and finally with ether for 2 weeks. The purified material was stored, still wet, at 0° with addition of toluene. A sample of this collagen was dried for 24 hours at 100° over P_2O_5.

1.182 gm. yielded 0.2191 gm. N, or 18.6%
1.351 " " 0.003 " ash as sulfate, or 0.22%

Determination of Glycine in Gelatin and Collagen--The determination of glycine with potassium trioxalatochromiate (7) does not lend itself to an evaluation from the point of view of the solubility product. The glycine compound precipitated contains glycine, potassium, and trioxalatochromiato ions in a rather complex ratio, rendering useless any mathematical treatment. It is possible, however, to apply to the glycine determination one of the checks worked out for the solubility method. By performing two comparative estimations, one with a certain amount of hydrolysate and the second with a smaller amount of hydrolysate to which is added a glycine solution of known content, one can detect in the determination any error exceeding 5 per cent. We feel justified, therefore, in accepting the results obtained with potassium trioxalatochromiate.

One precaution should be observed when potassium trioxalatochromiate is applied to the determination of glycine in protein hydrolysates. While the reagent has never been observed to precipitate any other natural amino acid except glycine, it does precipitate ammonia. The glycine content of the precipitate cannot, therefore, always be estimated solely by a determination of amino nitrogen. If considerable quantities of ammonia are present in the hydrolysate, part of it passes into the precipitate obtained with potassium trioxalatochromiate. The glycine content of this precipitate can be found by subtracting the ammonium nitrogen content of the precipitate from its total nitrogen content. The data from a series of determinations performed in this manner are found in Table II.

Determination of Proline in Gelatin and Collagen—50 cc. of a hydrolysate of pig skin gelatin (corresponding to 9.02 gm. of dry, ash-free protein) were mixed with 120 cc. of N HCl and the volume was made up with water to 1 liter. Three 275 cc. aliquots, each containing 2.48 gm. of protein, were added to solutions of 1.695, 2.001, and 2.301 gm. of ammonium rhodanilate, each dissolved in 175 cc. of methanol. The solutions were handled and the precipitates collected in the manner already described for the determination of pure proline, with the exception that these precipitates were not washed with water. The precipitates of *l*-proline rhodanilate weighed 1.283, 1.488, and 1.646 gm. The purity, determined by optical rotation, as described in the following paragraph, was 97.2, 96.9, and 96.7 per cent of the theory. From these data one may calculate, with the aid of Equation 2, the three values 442, 436, and 431 mg. for the *l*-proline content of the protein solutions used. The average, 436 mg., corresponds to 17.6 per cent of *l*-proline in pig skin gelatin. The results of all the determinations performed with varying amounts or concentrations of protein hydrolysate and with addition of proline are reported in Table III.

The purity of proline rhodanilate precipitates obtained in these determinations cannot be estimated accurately by elementary analysis alone. A more sensitive method has therefore been developed. Use was made of the fact that *l*-proline has a distinctively high negative rotation. The procedure was as follows:

A 300 mg. sample of a proline rhodanilate, the purity of which was to be ascertained, was shaken for ½ hour at room temperature with 1 cc. of glacial acetic acid and 0.15 cc. of dimethylaniline in a tared, stoppered flask. Then 2 cc. of water were added, the flask and contents were weighed and shaken vigorously, and the precipitated dimethylaniline rhodanilate was removed by filtration. The filtration was carried out under a difference in pressure of 6 mm. of Hg, with the use of the apparatus portrayed in Fig. 2. To the filtrate, which at this point was still colored, a few mg. of charcoal were added. The solution was then filtered again in the same way through a dry filter. The optical rotation of the filtrate was determined in a 1 dm. tube.

As a reference standard, pure proline rhodanilate was subjected to this procedure. 300 mg. of substance in 3.19 gm. of solvents gave α (1 dm.) = $-1.52°$ (average of eight determinations).

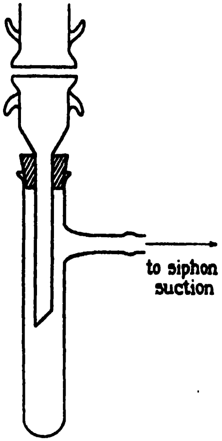

FIG. 2. Filter used in the decomposition of proline rhodanilate. A disk of hardened filter paper is inserted between the two halves of the filter, which are then clamped together by springs attached to the glass arms on either side.

In the calculation of the proline content of a proline rhodanilate sample obtained from the analytical procedures described, the following formula was used.

$$p = \frac{\alpha \times S \times 300 \times 57}{-1.52° \times 3.19 \times R}$$

where p = proline present (in mg.)
α = observed rotation (in degrees)
S = total weight of solvents (acetic acid, dimethylaniline, water, in gm.)
R = weight of sample of proline rhodanilate in mg.

300 mg. of pure proline rhodanilate correspond to 57.0 mg. of proline. The proline content of rhodanilate precipitates obtained in the course of analyses by the solubility method should closely correspond to this value. Determinations of the proline content of mixtures of proline rhodanilate and ammonium rhodanilate of known proportions indicated that the above procedure yields results accurate to ±1.5 per cent.

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MICRODETERMINATION OF ALKOXYL GROUPS

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A modified and improved apparatus and procedure for the quantitative microdetermination of alkoxyl groups are described. They are equally well adapted to solids, semiliquids, and liquids with boiling points above, at, or below that of hydriodic acid, irrespective of the number of alkoxyl groups. Accurate analytical figures are obtained by virtue of better solution of the substance, more gradual and longer heating, and thorough absorption.

The value of an accurate and generally applicable method for the microdetermination of the alkoxyl group is self-evident, yet many analysts report difficulties in the estimation of substances containing more than one such group.

In the course of thirteen years of microanalytical experience, many hundreds of alkoxyl determinations have been performed in this laboratory. The whole field has been investigated and improvements in technique and apparatus have been devised in order to overcome discrepancies and difficulties often reported.

The original Zeisel methoxyl macromethod, as adapted and modified for microdeterminations by Pregl (*13*), consisted in heating the substance with hydriodic acid, driving off the alkyl iodide with carbon dioxide, absorbing it in alcoholic silver nitrate, and estimating the silver iodide gravimetrically. Later Vieböck and Brecher (*16*) shortened the last part of the procedure by employing a volumetric titration of the iodine, which had distilled as alkyl iodide.

The modified apparatus of Rigakos (*14*) has been used in the author's laboratory since 1931. His improvement consists in the introduction of a ground-glass joint between the reaction flask and the outlet tube, and of a capillary leading from the side arm near the bottom of the reaction flask. Exactly the same modification of the apparatus was described much later by Neumann (*11*) for highly methylated carbohydrates. Clark has described (*3*) an apparatus having ground-glass joints and a capillary side arm, which can be used for macro- and microdeterminations. Chinoy (*2*) has also constructed a modified apparatus with a ground joint fitting into the reaction flask and with an extension ending in a glass ladle, which facilitates the introduction of the sample. These improvements have been applied

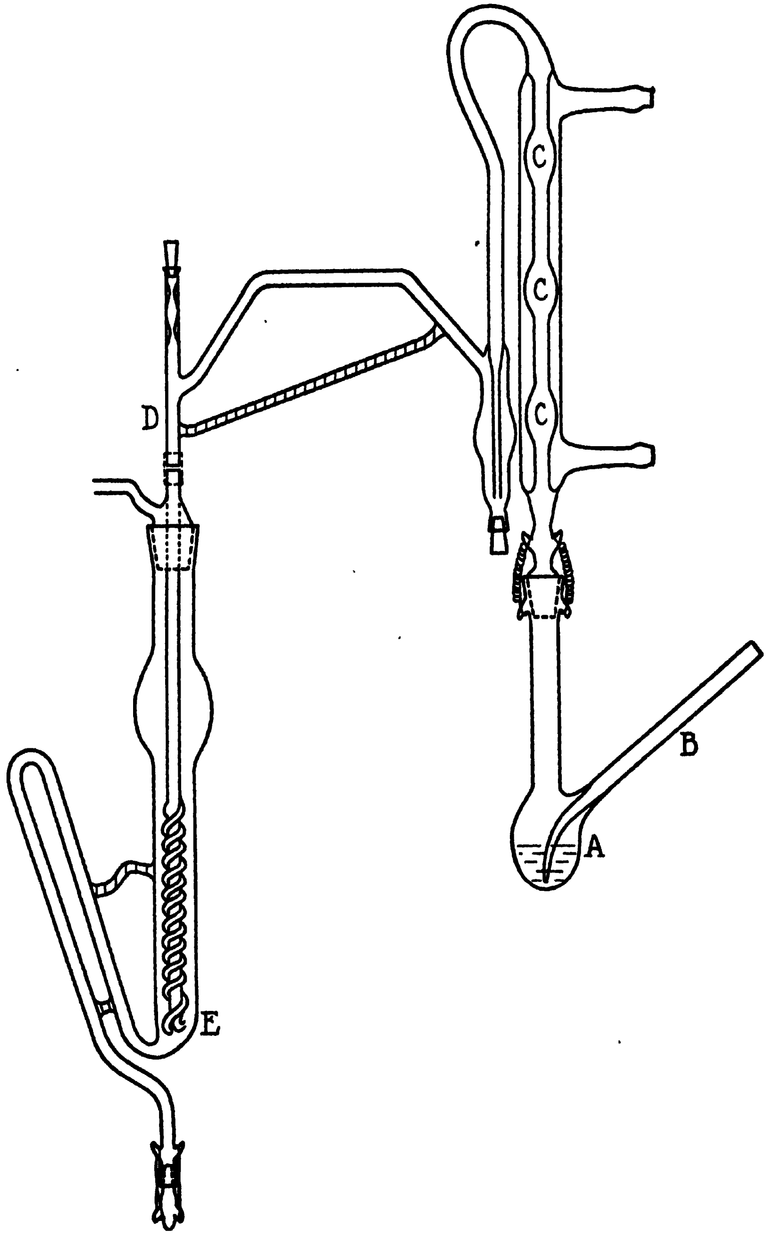

Fig. 1. Diagram of apparatus

to solid and sirupy substances and to liquids whose boiling points are above that of hydriodic acid.

Colson (*4*) has devised an apparatus for liquids having boiling points lower than that of the hydriodic acid, consisting of a vertical tube filled with small glass beads, which are wetted with hydriodic acid, connected above the reaction flask. He also connects in the train two washing tubes to entrap any hydriodic acid that might have distilled over because of its initial high specific gravity (1.96).

Lieff, Marks, and Wright (*10*) have described a modified apparatus which, they state, is more advantageous for the titration method. The Pregl type of receiver is replaced by an absorption tube containing four "pockets," the construction of which requires great care, and is attached to the outlet tube of the Pregl type of apparatus by a ground-glass joint in an inclined position.

Lately, in the author's laboratory an apparatus has been constructed (Figure 1) which has been found highly satisfactory for solids as well as for liquids with boiling points above, at, and below that of the hydriodic acid. There is a ground joint between the reaction flask and the outlet tube, as described by Rigakos (*14*). The ascending tube has three small bulbs, *C*, which unfailingly complete the condensation of any hydriodic acid, evaporated because of the heating, or possibly carried up mechanically by the carbon dioxide stream. These three bulbs, placed at suitable distances apart, also serve to cool the alkyl iodide entering the absorbent and eliminate the necessity of any cooling of the latter, such as recommended by White and Wright (*18*). Surrounding the bulbous portion of the ascension tube is a water condenser.

It is important in the determination of alkoxyl groups that there be thorough absorption, and, in the titrimetric method, complete oxidation of the alkyl iodide to the iodate. This is achieved by means of a long and slow passage of the alkyl iodide through the absorbing liquid. For this purpose a glass spiral, consisting of about twelve turns, was devised and attached to the outlet tube (*D*, Figure 1), which is immersed in the absorbent contained in a modified Kahovec (*8*) type of receiver. The bubbles enter the absorbent through a very small opening, *E*, and are forced to traverse the entire length of the glass spiral.

EXPERIMENTAL

Solid substances, previously dried and ground, are weighed by difference on the microbalance and placed on the bottom of the previously dried reaction flask, preferably using a long-handled charging tube.

For the weighing of semisolids, substances of sirupy consistency, and nonvolatile liquids, a small glass cup, about 4 to 5 mm. in diameter and height, which fits conveniently into the neck of the reaction flask, is used. This cup can also be used for solids.

A piece of glass in the shape of a semicylindrical boat or trough with open ends, made from glass tubing of suitable diameter by cutting it lengthwise, or a piece of platinum sheet molded to a boatlike shape also serves the purpose.

A few crystals of the purest phenol and 6 to 10 drops of acetic anhydride (or still better, propionic anhydride) are added and the flask is agitated in order to effect solution. If the substance is not completely dissolved the reaction flask is carefully heated in a water bath (or over a very small flame) to about 60° to 80°C., avoiding excessive heating. If complete solution still does not occur, a little more of the mixed solvent is added until all is dissolved, and the solution is then cooled. Since this extra solvent will considerably lower the eventual concentration of the hydriodic acid, 0.3 to 0.5 ml. extra of hydriodic acid (specific gravity, 1.96) must be added to restore its original specific gravity.

The reaction flask is then cooled, a few Alundum beads (size 16) are inserted, and about 2 ml. of hydriodic acid (specific gravity, 1.7) are added dropwise from a pipet, rotating the neck of the reaction flask to wash down any small particles of substance that might have adhered thereto. The washing tube has already been charged in the usual manner with 5 per cent cadmium sulfate solution and either a suspension of red phosphorus or a solution of sodium thiosulfate.

For the gravimetric determination, the usual Pregl type of receiver is charged with 2 ml. of alcoholic silver nitrate employing a straight outlet tube. For the more convenient volumetric determination of Vieböck and Brecher (*16*), the Kahovec (*8*) type of receiver is used, containing 4 to 5 ml. of glacial acetic acid–sodium acetate solution to which 6 to 8 drops of bromine are added. The glass spiral is carefully inserted into this solution, the reaction flask is connected as quickly as possible, and the side arm is attached to the source of carbon dioxide, the flow of which is reduced to a slow stream. The adjustment of the stream is best achieved by inserting between the side arm and the carbon dioxide generator a stopcock having a fine groove, similar to that used in the Dumas micromethod.

Solids and Nonvolatile Substances. The reaction flask is allowed to stand at room temperature for about 30 minutes, after which a small flame is placed under the flask and so regulated that the temperature of the solution remains below the boiling point for another 30 minutes. The flame is then raised slowly and the solution is brought to the boiling point, and maintained at that temperature for 1 to 2 hours—in some instances, even longer. About 15 minutes prior to the completion of the determination, the rate of carbon dioxide inflow is increased in order to drive traces of alkyl iodide into the receiver.

The gravimetric determination is performed in the usual manner. Using the titrimetric method, which is preferred, the receiver is emptied and its contents are washed into a ground-glass-stoppered Erlenmeyer flask (100 to 125 ml.) containing 0.5 to 1 gram of sodium acetate, which has been completely dissolved in a minimal volume of water (a 5 to 10 per cent sodium acetate solution may be used). The excess free bromine is now destroyed by adding a few drops of formic acid while whirling the flask several times. The flask is allowed to stand for a few minutes, then stoppered, and shaken well in order to remove the last traces of bromine from the vapor phase as well as from the solution. If, after this treatment, the solution is not completely decolorized, another drop of formic acid should be added with shaking. The usual amount of potassium iodide is added, the solution is acidified with 10 per cent sulfuric acid, the flask is again stoppered, and the solution is whirled. In case of a high methoxyl value, as evidenced by the intensity of the color of the iodine liberated, the standing time is extended to 10 to 15 minutes, instead of the usual 5 minutes, before titrating with the standard thiosulfate.

Volatile Liquids are weighed in a small glass cup with a ground-in stopper, or in a capillary tube about 2 mm. in width and 10 to 12 mm. in length. After the substance has been introduced into the capillary and centrifuged, the open end is sealed, and the tube is cooled and weighed.

The capillary is now cut just above the liquid level and both halves are dropped into the reaction flask, which has been previously charged with phenol, propionic anhydride, and a few granules of Alundum. Two milliliters of hydriodic acid are added and the flask is connected to the train as quickly as possible. The carbon dioxide stream is adjusted to a slow flow, and the reaction is allowed to proceed at room temperature for 30 to 40 minutes, with water running through the condenser.

A very small flame is then placed under the reaction flask and the heat gradually increased at such a rate that ebullition commences at the end of about an hour. Active boiling is maintained for a further period of about one hour, or even longer, in some cases of high methoxyl value.

The condenser is now emptied, and the carbon dioxide inflow is increased in order to drive the last traces of alkyl iodide into the absorbent, taking about 15 minutes to complete this procedure. The last step of the determination is the same as for nonvolatile substances.

DISCUSSION

The experiences of several investigators, with regard to the reliability of the alkoxyl-group microdetermination, seem rather divergent.

Friedrich (*5*) states that the method is very accurate if the hydriodic acid is pure, and that in some cases the substance must be brought into solution before adding the hydriodic acid; otherwise the methoxyl groups split only partially, or not at all. As a rule, it is sufficient to add the solvent.

Ware (*17*) reports that, if the substance contains more than two methoxyl groups, the hydriodic acid (specific gravity, 1.7) should be replaced by hydriodic acid (specific gravity, 1.96) in order to get reliable figures, and she uses a correction of 0.12 mg. for 2 ml. of the alcoholic silver nitrate solution as suggested by Friedrich (*6*).

According to Bruckner (*1*) the claim of Ware is actually due to the incomplete solution of special substances in phenol and acetic anhydride, or in some instances, phenol and propionic anhydride. He erroneously thinks that the quantitative splitting of the methoxyl by the hydriodic acid is not related to the number of the methoxyl groups, but rather to their position in the molecule and to the specific behavior of the substance.

In the case of highly methylated carbohydrates, and frequently with well-known crystalline methylated sugars, Neumann (*11*) mentions that methoxyl values which are 1 to 2 per cent low have often been reported, and attributes this discrepancy to the fact that the reaction flask is heated over a free flame and the substance in the flask is more or less "caked." According to him, this caked portion of the substance does not react with the hydriodic acid, and the variation in the degree of caking is responsible for the nonreproducibility of values obtained on the same sample, especially with polymeric carbohydrates. He, therefore, employs an oil bath with gradual elevation of the temperature in order to circumvent this effect.

The modified apparatus and procedure described in this paper are calculated to ensure greater accuracy in the alkoxyl microdetermination. Complete solution of the substance is one of the requisites for correct results. Caking does not take place if the substance is properly dissolved.

Bruckner (*1*) obtained correct data in some cases without the use of any solvent, but it is generally agreed that complete solution of the substance is necessary. Pregl (*13*), Niederl (*12*), and Kuhn and Roth (*9*) mention solubility tests before the addition of the hydriodic acid, but this is obviously not feasible when only a small amount of substance is available. The safest procedure to follow, as found in this laboratory, is to add phenol and propionic anhydride, which are superior to phenol and acetic anhydride. The latter combination has been used in many instances with a longer period of boiling without giving correct analytical figures. The data in Table I substantiate the claim as to the efficacy of phenol and propionic anhydride.

Another important part of the author's procedure is to allow the reaction to proceed at room temperature, particularly when one or more of the alkoxyl radicals are in an ester form. Since the first product of the reaction will then be methanol, this might distill over, after the application of heat, without being converted into methyl iodide. The same condition obtains for volatile liquids; hence the advantage of having a condenser above the reaction flask. The expenditure of time for heating is well worth while, because it effects complete severance of the alkoxyl groups, irrespective of their number. Inasmuch as the procedure requires only occasional attention, the increase in time is not significant.

The disturbing occurrence of bumping so often discussed is best prevented by the use of a few Alundum grains.

When high methoxyl figures are anticipated it has been found necessary to have an excess of bromine in the absorbent, lest the alkyl iodide be incompletely oxidized, and the weight of the sample should be close to 3 mg., to ensure a sufficient excess of hydriodic acid.

According to Roth and Daw (*15*) the method using alcoholic silver nitrate as an absorbent is not applicable to sulfur-containing substances because the cadmium sulfate in the washer does not retain quantitatively the hydrogen sulfide liberated during the reaction. It has been the experience in this laboratory that by the addition of 1 or 2 crystals of iodine to the reaction mixture, the organically bound sulfur is oxidized practically quantitatively to the elementary form. Therefore, only a trace (if any) of hydrogen sulfide is formed, and this is easily retained by the cadmium sulfate. However, this is not a factor in the more generally employed volumetric method of Vieböck and Brecher (*16*).

A blank of 0.02 to 0.30 ml. has been found by Gibson and Caulfield (*7*) and Lieff, Marks, and Wright (*10*), depending on the purity of the reagents. If the best available chemicals are used the blank is negligible (*16*).

As shown in Tables I, II, and III, when the above described procedure is followed the results are equally accurate for gravimetric or volumetric determinations, and for solids, sirupy substances, and liquids with boiling points at, above or below that of the hydriodic acid. Duplicate analyses illustrate that the precision of the method is high. The data in Table IV were obtained independently (by J. A. Alicino, Department of Chemistry, Fordham University, New York, N. Y.) using the conventional procedure. The low figures

TABLE I

Determination of Methoxyl

No.	Compound	Formula	Methoxyl Groups per Molecule	Weight of Sample	Vol. of 0.01 *N* $Na_2S_2O_3$ (Cor.)	Methoxyl Found	Methoxyl Calculated
				Mg.	*Ml.*	%	%
1	Heptaacetyl N-acetyl chondrosin methyl ester	$C_{29}H_{41}O_{19}$	1	3.795	3.19	4.11	4.09
2	Methyl ester of diacetonegalacturonic acid	$C_{13}H_{20}O_7$	1	3.982	8.32	10.79	10.76
3	Pentamethyl methylgalacturonidorhamnoside methyl ester	$C_{19}H_{34}O_{11}$	7	3.227	30.92	49.57	49.54
4	Methylglycoside of hexamethylaldobionic acid methyl ester	$C_{20}H_{36}O_{12}$	8	2.719	27.60	52.68	53.00
5	Methylglycoside of hexamethyl-6-glucosidogalactose	$C_{19}H_{36}O_{11}$	7	3.132	29.77	49.30	49.30
6	Methylglycoside of heptamethyl-6-glucosidogalactose	$C_{20}H_{38}O_{11}$	8	3.001	31.91	54.97	54.60
7	Nonamethyl glucosidosorbitol	$C_{21}H_{42}O_{11}$	9	3.497	39.41	59.08	59.32
8	Pentamethylsorbitol	$C_{11}H_{24}O_6$	5	3.341	39.70	61.44	61.44
9	Pentamethylsorbitol	$C_{11}H_{24}O_6$	5	2.695	31.93	61.25	61.44
10	Tetramethyl methylglucoside	$C_{11}H_{22}O_6$	5	2.914	34.88	61.88	62.00

TABLE II

Determination of Methoxyl

No.	Compound	Formula	Boiling Point	Methoxyl Groups per Molecule	Weight of Sample	Vol. of 0.01 *N* $Na_2S_2O_3$	Methoxyl Found	Methoxyl Calculated
			°*C.*		*Mg.*	*Ml.*	%	%
11	Trimethyl xylulose	$C_8H_{16}O_5$	64 at 0.25 mm. Hg	3	3.458	32.36	48.38	48.45
12	Trimethyl xylulose	$C_8H_{16}O_5$	64 at 0.25 mm. Hg	3	2.789	26.41	48.36	48.45
13	Trimethyl methyl-*d*-xyluloside	$C_9H_{18}O_5$	52 at 0.25 mm. Hg	4	3.036	35.73	60.10	60.21
14	Dimethyl monoacetone *d*-xylulose	$C_{10}H_{18}O_5$	47 at 0.1 mm. Hg	2	3.457	19.31	28.29	28.44
15	Dimethyl xylulose	$C_7H_{14}O_5$	88 at 0.2 mm. Hg	2	3.292	22.20	34.55	34.84
16	Dimethyl methyl-*d*-xyluloside	$C_8H_{16}O_5$	61 at 0.25 mm. Hg	3	2.955	27.93	48.27	48.45
17	5-Methyl monoacetone · methylrhamnoside	$C_{11}H_{20}O_5$	63 at 0.1 mm. Hg	2	3.480	17.92	26.62	26.72

TABLE III

Determination of Methoxyl

No.	Compound	Formula	Boiling Point	Methoxyl Groups per Molecule	Weight of Sample	AgI	Methoxyl Found	Methoxyl Calculated
			°C.		Mg.	Mg.	%	%
18	Trimethyl methyl-*d*-xyluloside	$C_9H_{18}O_5$	52 at 0.25 mm. Hg	4	3.600	16.390	60.09	60.21
19	Dimethyl monoacetone xylulose	$C_{10}H_{18}O_5$	47 at 0.1 mm. Hg	2	3.573	7.716	28.52	28.44
20	Dimethyl xylulose	$C_7H_{14}O_5$	88 at 0.2 mm. Hg	2	4.184	10.950	34.57	34.84
21	Dimethyl methylxyluloside	$C_8H_{16}O_5$	61 at 0.25 mm. Hg	3	3.308	15.933	48.15	48.45
22	Triacetylmethyl methylhexoside	$C_{14}H_{22}O_9$	....	2	4.775	6.685	18.48	18.55
23	Diacetyl dibenzoyl methylhexoside	$C_{25}H_{26}O_2$	....	1	5.875	2.831	6.36	6.38
24	α-Methyl-2,3,4-trimethyl-*d*-galacturonamide	$C_{10}H_{19}O_6N$	....	4	3.182	12.029	49.94	49.81
25	Dimethyl ester of 2,3,4-trimethoxy mucic acid	$C_{11}H_{20}O_8$	....	5	3.189	13.305	55.11	55.37

TABLE IV

Determination of Methoxyl

No.	Compound	Formula	Methoxyl Groups per Molecule	Weight of Sample	Vol. of 0.02195 *N* $Na_2S_2O_3$	Methoxyl Found	Methoxyl Calculated
				Mg.	Ml.	%	%
26	Trimethyl methylglucoside	$C_{10}H_{20}O_6$	4	3.974	13.70	39.04	52.54
27	Trimethyl methylglucoside	$C_{10}H_{20}O_6$	4	3.750	14.30	43.19	52.54
28	Trimethyl methylglucoside	$C_{10}H_{20}O_6$	4	4.975	14.87	33.85	52.54
29	Trimethyl methylglucoside	$C_{10}H_{20}O_6$	4	4.810	16.95	39.91	52.54
30	Trimethyl methylglucoside	$C_{10}H_{20}O_6$	4	4.520	15.60	39.09	52.54
31	Trimethyl methylglucoside	$C_{10}H_{20}O_6$	4	4.395	16.65	42.90	52.54
32	Hexamethylsorbitol	$C_{12}H_{26}O_6$	6	4.480	20.80	52.70	69.87
33	Hexamethylsorbitol	$C_{12}H_{26}O_6$	6	4.920	22.17	51.03	69.87
34	Hexamethylsorbitol	$C_{12}H_{26}O_6$	6	4.170	21.50	58.39	69.87
					0.01993 *N* $Na_2S_2O_3$		
35	Trimethyl monoacetone glucose	$C_{12}H_{22}O_6$	3	6.890	18.05	27.00	35.50
36	Trimethyl monoacetone glucose	$C_{12}H_{22}O_6$	3	5.740	14.70	26.40	35.50

(in some instances 10 to 15 per cent below the calculated value) and the inconsistencies for pure substances, which were checked by carbon and hydrogen determinations, are evident. Table V presents very satisfactory figures obtained (by Alicino) following the procedure now described (using information given in advance of publication by the present author).

TABLE V

Determination of Methoxyl

No.	Compound	Formula	Methoxyl Groups per Molecule	Weight of Sample	Vol. of 0.01064 *N* $Na_2S_2O_3$	Methoxyl Found	Methoxyl Calculated
				Mg.	*Ml.*	%	%
37	Trimethyl methylglucoside	$C_{10}H_{20}O_6$	4	3.348	32.06	52.70	52.54
38	Trimethyl methylglucoside	$C_{10}H_{20}O_6$	4	2.902	27.70	52.53	52.54
39	Trimethyl monoacetone glucose	$C_{12}H_{22}O_6$	3	2.882	18.63	35.58	35.50
40	Trimethyl monoacetone glucose	$C_{12}H_{22}O_6$	3	2.822	18.17	35.44	35.50
41	Hexamethylsorbitol	$C_{12}H_{26}O_6$	6	2.106	26.62	69.57	69.87
42	Tetramethylsorbitol	$C_{10}H_{22}O_6$	4	2.618	25.02	52.60	52.03
43	Hexamethylmannitol	$C_{12}H_{26}O_6$	6	2.028	25.74	69.85	69.87

The procedure and apparatus are the same for both methoxyl and ethoxyl groups.

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DELPHININE

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The seeds of *Delphinium staphisagria*, L., have been found on extraction to yield an appreciable alkaloid fraction only a portion of which has been obtained in crystalline form. This appears to consist essentially of the alkaloid delphinine. Our knowledge of the chemistry of the latter, as in the case of the alkaloids contained in other *Delphinium* species and the unquestionably closely related aconite alkaloids, is but in the earliest stage. This may be briefly summarized as follows: Walz (1) revised the previous formulations proposed by earlier workers to $C_{34}H_{47}O_9N$ on the basis of the analysis of the alkaloid itself and of its oxalate. This formula has been supported by Keller (2) and by the analyses reported by Markwood (3). The earlier work of Katz (4) had shown the alkaloid to contain methoxyl and to yield benzoic acid on saponification. Subsequently Walz and Keller confirmed the presence of four methoxyl groups and one benzoyl group. On the basis of acyl determinations on an amorphous "acetyl delphinine" and "propionyl delphinine," Walz concluded that at least one acylatable hydroxyl group is present in the alkaloid. Thus, in the four methoxyl groups and the benzoyl and hydroxyl groups, 7 of the 9 oxygen atoms of delphinine appeared to be accounted for.

As a possible aid in the approach to the chemistry of the aconite alkaloids, we have recently included the *Delphinium* alkaloids in our studies, since they appear to contain fewer of the troublesome oxygen atoms. The delphinine prepared by us from commercial *staphisagria* seeds was found to agree essentially with the properties already recorded for this alkaloid. However, our analytical results have been consistently in closer agreement with the figures required by a modified formula, *viz.* $C_{33}H_{45}O_9N$. This formula was also supported by the analysis of the *hydrochloride* of delphinine. Our analyses have confirmed the presence of four methoxyl groups in the alkaloid.

A study of the saponification of the alkaloid with alkali and titration of the liberated volatile acids has shown that two acid groups are removed in the process. The liberation of benzoic acid was confirmed but in addition acetic acid was found to be present and was isolated as the silver salt. Experiments which are still in progress, and which will be reported in another connection, have indicated the presence of a hydroxyl group.

Thus the functions of the 9 oxygen atoms of the alkaloid are defined by the four methoxyl groups, two acyl groups, and one hydroxyl group. On catalytic hydrogenation 3 moles of hydrogen were absorbed by the alkaloid and a crystalline *hexahydrodelphinine* resulted. Since the latter on saponification no longer yielded benzoic acid but hexahydrobenzoic acid instead, the alkamine portion of the molecule was not hydrogenated. This parallels the experience with aconitine (5) and suggests a saturated character for the parent alkamine.

In addition to the four methoxyl groups, delphinine has now been found to behave as if it contained an N-alkyl group. When the alkaloid was submitted to alkali fusion, methylamine was isolated from the volatile material and was identified as the picrate. If the group in question is an N-alkyl group, it must therefore be methyl. Hence there is a close parallelism in the pictures presented by delphinine and the aconite alkaloids. In the case of the latter, aconitine $C_{34}H_{47}O_{11}N$ (6, 7) behaves as if an N-ethyl group is present in addition to four methoxyl groups, three hydroxyl groups, a benzoyl, and an acetyl group. In hypaconitine, $C_{33}H_{45}O_{10}N$, and mesaconitine, $C_{33}H_{45}O_{11}N$, the N-alkyl group has been shown to be methyl (7).

Another observation which parallels the experience with aconitine is the so called "X-214°," a substance obtained by Keller (2) by the oxidation of delphinine with permanganate and in which the salt-forming properties of delphinine have been lost. A provisional formula of $C_{28}H_{37}O_9N$ was derived for it. We have confirmed the production of this substance and the possibility is at once suggested of its analogy to oxonitine from aconitine. This is supported by the fact that just as oxonitine no longer contains the N-ethyl group (or equivalent) of aconitine, this oxidation product likewise no longer possesses the N-methyl group (or equivalent) of delphinine. Titration after saponification has shown the liberation of two acid groups and therefore the retention of the acetyl and benzoyl groups of delphinine, just as it has been found to be the case with oxonitine. It is most likely a lactam. A discussion of the exact formulation of this substance we wish to leave to a later occasion.

EXPERIMENTAL

Delphinine—The alkaloid fraction was obtained from the powdered seeds essentially as described by Markwood. The concentrated ligroin extract of the seeds was in turn exhaustively extracted with 3 per cent tartaric acid. During the first few extractions with the latter, calcium tartrate crystallized during the process and was removed by filtration. Later extractions were not complicated by this crystallization and the process was continued as long as the acid extract gave a precipitate with alkali. The acid extracts were cleared by shaking out successively with petroleum ether and ether and were then made alkaline. The precipitated alkaloid was reextracted with ether. The latter on concentration gave a copious basic oil in which crystals of delphinine formed. After standing the alkaloid was collected with a mixture of ether and petroleum ether. The yield approximated 0.8 gm. from 1 kilo of seeds. After recrystallization from alcohol the alkaloid formed six-sided plates which melted at 198–200°. Keller and Walz, as well as Markwood, have given the uncorrected melting point of 187.5°, and Stojanow the corrected figure 191.8°. We have found $[\alpha]_D^{25} = +25°$ ($c = 1.215$ in absolute alcohol). Keller gave $[\alpha]_D^{20} = +18.99°$ for a solution which had stood for 4 hours. He also reported a rotation taken shortly after solution of the alkaloid from which can be calculated a rotation of $[\alpha]_D^{20} = +22°$ ($c = 0.86$ in absolute alcohol). This value is comparable with our own.

$C_{33}H_{45}O_9N$.	Calculated.	C 66.07, H 7.57, N 2.34
$C_{34}H_{47}O_9N$.	"	" 66.52, " 7.72, " 2.28
	Found. (*a*)	" 66.04, " 7.52
	" (*b*)	" 66.00, " 7.61
	" (*c*)	" 65.94, " 7.61
$C_{33}H_{45}O_9N$.	Calculated.	4(OCH_3) 20.71, (N)CH_3 2.51
	Found.	" 20.58
	"	" 20.45, " 2.62

15.295 mg. of delphinine were refluxed in 3 cc. of 0.1 N NaOH and 3 cc. of alcohol for 3 hours and titrated back against phenolphthalein. Found, 0.347 cc. Calculated for 2 equivalents, 0.51.

15.650 mg. were refluxed as above, but for 7 hours. Found, 0.345 cc. Calculated for 2 equivalents, 0.522.

Since the correct titration of the base was undoubtedly affected by the basicity of the alkamine cleaved during the saponification, the following procedure was used.

0.4255 gm. of delphinine was refluxed in a mixture of 10 cc. of N NaOH and 10 cc. of methanol for 2.5 hours and protected as usual from the air. The mixture was acidified with 5 cc. of 25 per cent H_2SO_4 and then distilled with steam. The distillate was titrated with 0.1 N NaOH and was collected as long as the distillate consumed alkali. The total alkali used was 13.66 cc. Calculated for 2 equivalents, 13.88 cc.

The titration mixture was concentrated *in vacuo* and in a volume of about 2 cc. was acidified with dilute HNO_3. Precipitated benzoic acid was collected with water. After recrystallization from petroleum ether it melted at 121°.

$C_7H_6O_2$. Calculated, C 68.82, H 4.96; found, C 68.70, H 5.02

The above filtrate from the crude benzoic acid was extracted several times with small amounts of petroleum ether to remove dissolved benzoic acid and then carefully neutralized with dilute NaOH. After concentration to a small volume the mixture was treated with strong silver nitrate solution. The collected silver salt was recrystallized from dilute alcohol.

$C_2H_3O_2Ag$.	Calculated.	C 14.38,	H 1.81,	Ag 64.64
	Found.	" 14.62,	" 1.89,	" 64.72

Delphinine Hydrochloride—The salt was readily obtained by addition of dry ether to the concentrated solution of the alkaloid in methanol containing an excess of HCl. It formed needles from methanol and ether, which melted at 208–210°, depending upon the rate of heating.

For analysis it was dried at 120° and 15 mm.

$C_{33}H_{45}O_9N \cdot HCl$.	Calculated.	C 62.28,	H 7.29
	Found.	" 62.16,	" 7.47
	"	" 62.40,	" 7.68

Hexahydrodelphinine—0.11 gm. of delphinine was dissolved in a few cc. of alcohol containing 0.2 cc. of acetic acid, and hydrogenated with 50 mg. of platinum oxide catalyst under a pressure of 3 atmospheres. Absorption was prompt and stopped after about 30 minutes. The absorption due to the substance was 12.6 cc. of H_2 or approximately 3 moles. After removal of the solvent the substance crystallized readily under ether. After recrystallization from this solvent it melted at 192–193°.

$C_{33}H_{51}O_9N$. Calculated, C 65.41, H 8.49; found, C 65.33, H 8.21

On saponification this substance gave no benzoic acid but an oily acid which from its odor and properties was unquestionably hexahydrobenzoic acid.

Alkali Fusion of Delphinine—A mixture of 1 gm. of the alkaloid with 5 gm. of powdered KOH was heated in a Pyrex cylindrical vessel immersed in a nitrate bath. Hydrogen was passed through during the operation and volatile material was collected in a bent tube which formed the outlet. The gases were continued through wash bottles containing dilute HCl. Copious fumes were evolved and a yellow, viscous resin began to distil with the bath at 260°. The operation was held at this point for 30 minutes and was then discontinued. The yellowish resin was in large part dissolved by dilute HCl which rapidly assumed a violet color. This mixture was joined with the aid of alcohol with the contents of the wash bottles and was then extracted repeatedly with ether. The aqueous phase was concentrated *in vacuo* to dryness, leaving a partly crystalline residue. On addition of a little water a resin remained, which was removed by filtration. The filtrate and washings were made alkaline and distilled with steam into dilute HCl. The latter on evaporation gave 9 mg. of a crystalline residue.

This salt was decomposed under ether with NaOH. The ethereal solution which smelled strongly of methylamine was dried over KOH. On addition of ethereal picric acid solution a deposit of needles at once formed. After collection it was recrystallized from alcohol ether and melted at 205–207°.

$C_7H_8O_7N_4$. Calculated, C 32.31, H 3.11; found, C 32.41, H 3.01

Oxidation of Delphinine—1.05 gm. of the alkaloid dissolved in 100 cc. of dry acetone were treated with 1.1 gm. of $KMnO_4$ and the mixture was occasionally shaken. Several days at room temperature were required before the reagent was used up. Since crystals of the oxidation product appeared on the walls of the vessel, more solvent was added and the mixture was warmed to dissolve them. After filtration the MnO_2 was again extracted with warm solvent. On concentration of the filtrate to small bulk successive fractions of the product were obtained, which amounted to 0.49 gm.

For purification it was dissolved in chloroform and shaken out with dilute H_2SO_4 to remove any traces of unchanged alkaloid or other basic material. The concentrated solvent readily crystallized on addition of alcohol. Flat needles separated, which melted at 218–220°. Occasionally a melting point of 225° was observed. The apparently neutral character of the substance was confirmed.

For analysis the substance was dried at 100° and 15 mm.

Found.	C 64.58,	H 7.00,	OCH_3 19.60,	$(N)CH_3$ 0.64
"	" 64.70,	" 6.90		
"	" 64.82,	" 7.04,	" 19.38,	" 0.63

Titration showed the retention of the benzoyl and acetyl groups in this substance.

14.285 gm. of substance were refluxed in 3 cc. of 0.1 N NaOH and 3 cc. of alcohol for 4 hours and then titrated back against phenolphthalein. Found, 0.438 cc. For an approximate molecular weight of 600 the calculated value for 2 equivalents is 0.47 cc.

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A MODIFICATION OF THE SCHLIEREN METHOD FOR USE IN ELECTROPHORETIC ANALYSIS

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The scale and schlieren methods have both been used for the determination of the refraction gradients that arise in electrophoretic and ultracentrifugal analysis.[1, 2] With proper conditions either method yields a graph of the gradient, dn/dx, in a thin horizontal layer of the column as a function of the position, x, of the layer. With the scale method, however, this graph is obtained by a laborious comparison of the scale photographs. The schlieren method may be modified to record this graph rapidly and automatically as will be shown below.

The schlieren method, as applied to electrophoretic analysis, may be described with the aid of Fig. 1. An image of the horizontal slit S, illuminated by the lamp L and condenser C, is formed by the lens D in the plane P. An opaque diaphragm with a sharp horizontal upper edge is placed in this plane and may be moved vertically. The camera objective O is focussed on the electrophoresis cell E and forms an image of this on the photographic plate G. If refraction gradients, *i.e.*, electrophoretic boundaries, are present in the cell E the pencils of light through these gradients are deflected downward. With the diaphragm adjusted to intercept these deflected pencils, the regions at G conjugate to the boundaries in the cell appear as dark "schlieren" bands. Using corrected lenses and a narrow slit S the edges of the

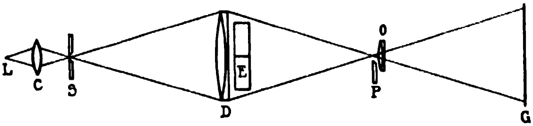

FIG. 1

schlieren bands are quite sharp. The displacement of the diaphragm from the position of the undeviated slit image is proportional to the refraction gradient at positions in the cell E conjugate to the edges of the schlieren bands.

The modification of the schlieren method I have used consists in masking the cell image at the photographic plate by a narrow vertical slit and driving the plate horizontally past this slit as the diaphragm is progressively raised to the position of the undeviated slit image. One thus obtains on the plate a transparent area whose contour is a graph of the refraction gradient dn/dx *versus* the position x. Figures 2 and 3 (positive prints) were obtained in this manner. Figure 2 was obtained in the electrophoresis of a 0.5% solution of an egg albumin preparation and indicates the presence of an impurity to the extent of about 25% of the total protein. Figure 3 was obtained with horse serum (diluted one to four) and indicates a marked overlapping of the gradients corresponding to the albumin and the α-, β- and γ-globulins.[3]

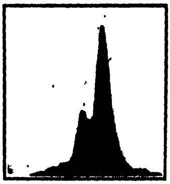

FIG. 2

In obtaining the records illustrated by Figs. 2 and 3 the plate was geared to travel 7.5 times as fast as the diaphragm. The lenses D and O, Fig. 1, had 36″ (91-cm.) focal lengths, the aperture ratio of the latter was F/36 and unit magnification was used. With a 0.2 × 25 mm. slit illuminated by an "H4" mercury lamp and a 0.2 mm. masking slit a plate travel of 15.1 mm. per minute adequately exposed an Eastman contrast lantern slide. Thus only about three minutes were required to make the exposures.

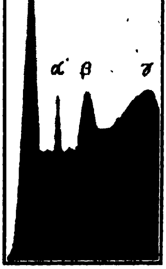

FIG. 3

The modification of the schlieren method outlined here has an advantage over that described by Philpot[4] in that the position of the base line is definite even in the presence of linear gradients in the column. Moreover, the method is rapid and flexible in its application. The quantitative comparisons that have been made indicate that the precision attainable is comparable with that of the scale method. As Philpot has suggested, the photographic record thus obtained lends itself readily to a direct photometric determination of the area under the contour and hence of the protein concentration.

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(2) Tiselius, *Särtryck Svensk Kem. Tidskrift*, **50**, 58 (1938).
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ELECTROPHORESIS OF PROTEINS BY THE TISELIUS METHOD[1]

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(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, January 30, 1939)

INTRODUCTION

The recent improvements introduced by Tiselius (15) in the electrophoretic method have greatly increased its importance in the study and preparation of proteins. Our contribution to this Symposium will consist of a discussion of the principles underlying the Tiselius method, together with a description of the experimental procedure involved in the use of the method, including such modifications as we have introduced. The topics to be considered are (*a*) the apparatus, (*b*) the optical principles involved in the observation of electrophoretic boundaries, (*c*) the effects of thermal convection, (*d*) some typical applications of the method, and (*e*) "boundary anomalies". These will be treated in the order given.

APPARATUS

To study electrophoresis of proteins and related materials by the moving boundary method it is necessary, in the first place, to form a boundary between a solution of the material in a suitable buffer and the buffer itself. Passage of current then causes the boundary to move. For this movement to be a measure of the mobility of the protein it is desirable, first, that the electric field and pH in the neighborhood of the boundary be substantially constant, second, that the electrode processes do not involve the evolution of gas or other uncertain volume changes, and third, that the electrode products do not reach the regions in which the boundaries are moving. These conditions have been admirably met in the electrophoretic apparatus developed by Tiselius, which, with the modifications that we have made, will be described below.

The cell in which the boundaries are formed and observed is shown in cross section in figure 1A and consists of the sections I, II, III, and IV.

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[1] Presented at the Symposium on the Physical Chemistry of the Proteins, held at Milwaukee, Wisconsin, September, 1938, under the auspices of the Division of Physical and Inorganic Chemistry and the Division of Colloid Chemistry of the American Chemical Society.

These may be slid over one another along the planes a-a′, b-b′, and c-c′. Through the cell runs a U-shaped channel d-d′ of rectangular cross section. Figure 1B is a top view of one of the center sections. To form a boundary, the channel is filled with the buffer solution of protein to a level slightly above the plane b-b′, about 10 ml. of solution being required. Section III is then pushed to one side, the excess solution in section II is removed, and this section is rinsed with buffer. The remainder of the cell and the attached electrode vessels to be described are then filled with the buffer.

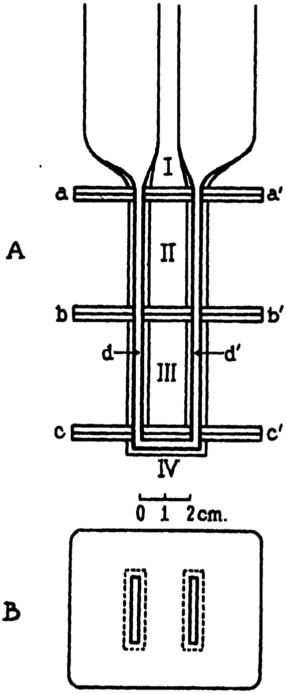

Fig. 1. A, electrophoresis cells in cross section; B, top view of one of the center sections.

The support for the cell and the electrode vessels, together with the mechanism for moving the sections of the cell in relation to each other, are shown in figure 2. In our apparatus a rack-and-pinion system replaces the pumps utilized by Tiselius for this purpose. Turning the knurled knobs k-k′ attached to the concentric shafts (s) operates the bevel gears (g), which in turn cause a horizontal motion of the racks r. Each rack presses against a metal insert e, which communicates the pressure to the edges of the horizontal glass plates. The sections II and III may therefore be shifted in either direction by manipulation of the appropriate knurled knob.

After filling the apparatus as described above, the silver-silver chloride electrodes (E-E′ of figure 2) are inserted. For effective operation of these electrodes, described more fully later, they must be immersed in a strong chloride solution. This is accomplished by carefully introducing the solution through the silver tubes t-t′. These silver tubes, which are insulated by the glass tubes (j), also serve as current leads to the electrodes E-E′. We have modified the procedure of Tiselius and our earlier procedure, in that one side of the apparatus is closed, care being taken to exclude air bubbles. This is accomplished with the ground-glass stopper f and the stopcock m. Closing one side is more convenient than having both sides open, as the latter procedure involves equalizing the liquid levels on the

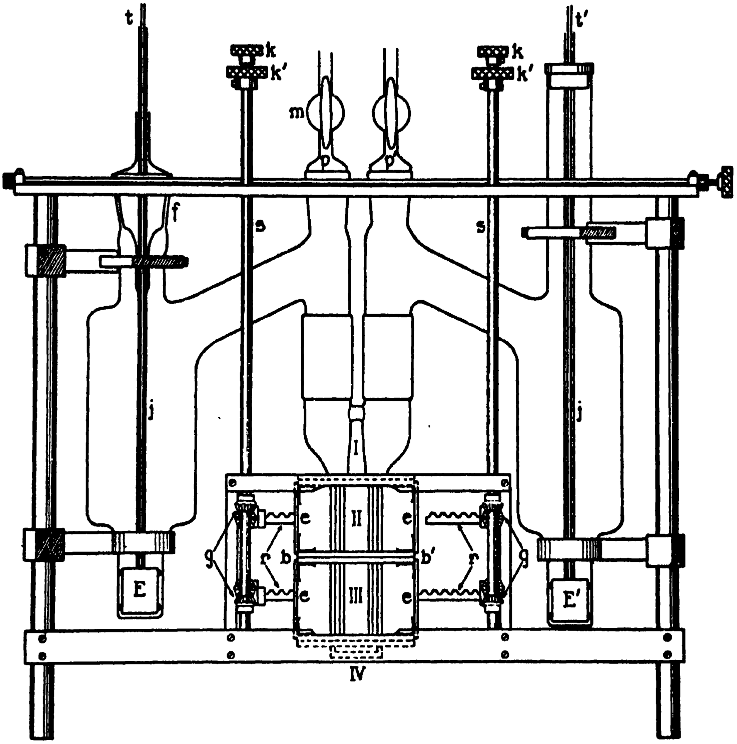

FIG. 2. Electrophoresis cell, electrode vessels, and support

two sides of the system before forming the boundaries. It also permits the use of an improved type of compensation, to be described below.

With the cell and electrode vessels filled as described, the two boundaries are formed in the plane b-b′ by returning section III to the position shown. A potential from a battery or "power pack" applied to the terminals t-t′ will then, in general, cause the boundary in one side of the cell to rise and that in the other to fall.

With the design of the apparatus shown in figure 2, filling of the cell and electrode vessels, as described, may be carried out in the low-temperature thermostat. Since the stoppers p and p′ are directly above the cell, the protein solution and buffer may be introduced with pipets. For this purpose we have found a syringe, provided with a long stainless-steel needle, serviceable.

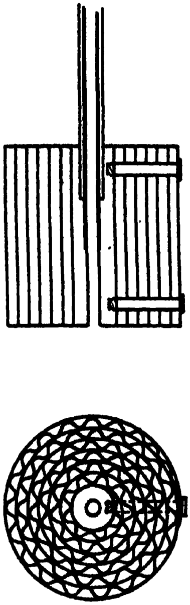

FIG. 3. Diagram illustrating the electrode construction

Further details of the electrodes E-E′ are as follows. They must be capable of carrying currents of at least 30 milliamperes for long periods of time without evolution of gas. Sufficient capacity has been obtained by winding a flat and a corrugated strip of sheet silver together into a tight spiral, as shown in figure 3. The ends of the spirals are anchored to a hollow silver core with silver screws. The silver tube is also threaded into this core. This type of construction, suggested by Professor H. P. Cady, exposes a large electrode surface to the electrolyte. Air bubbles trapped by the electrodes may be easily dislodged.

OBSERVATION OF THE BOUNDARIES

Another important contribution to the electrophoretic method made by Tiselius was the adaptation of Toepler's "schlieren" (shadow) method (9) for the observation of the boundaries. A diagram of the optical system is shown in figure 4. The image of the slit S, illuminated by the lamp L and condenser C, is brought to focus in the plane P by the lens D. The schlieren diaphragm, a screen with a sharp, horizontal upper edge, is placed in the plane P and may be moved vertically, a micrometer adjustment being used. The cell E, in which the electrophoresis is carried out, is placed as near the lens D as the thermostat construction permits. The camera objective O, placed immediately behind the schlieren diaphragm, is focussed on the cell and forms a full-size image on a ground-glass or photographic plate at G. Further details of the system as used in obtaining the results described in this paper are indicated in the legend of the figure.

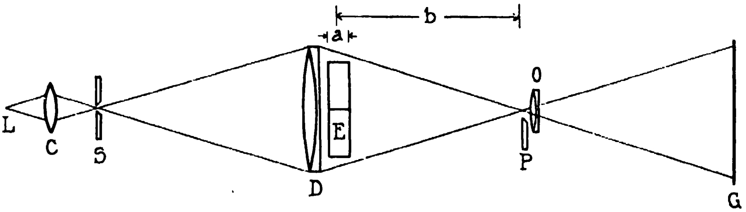

FIG. 4. Arrangement for observation of the boundaries with the schlieren method. L, 5-volt, 18-ampere ribbon filament lamp; C, projection condenser; S, 0.3 × 15 mm. horizontal slit; D, Dallmeyer portrait lens 6 D, 4 in. diameter, 21 in. focal length; E, electrophoresis cell; P, schlieren diaphragm; G, ground-glass screen or photographic plate; O, Dallmeyer R.R. lens, 3 in. diameter, 24 in. focal length.

In the absence of refraction gradients in the electrophoresis cell all of the light is brought to focus in the image of the illuminated slit at P and enters the camera objective. If, however, a boundary is present in the cell, the refraction decreases with increasing height through the boundary, and the pencils of light through this region are deflected downward. These deflected pencils are intercepted by the schlieren diaphragm and fail to reach the screen. Thus the region at G conjugate to the boundary appears as a dark band on a light background. This is shown in figure 5. The horizontal dark lines are the schlieren bands of the boundaries between a

0.5 per cent egg albumin solution in a 0.02 normal sodium acetate buffer at pH 5.2 and the pure buffer. The upper and lower photographs are of the boundaries migrating in the anode and cathode sides of the channel, respectively. Exposures were made at 30-min. intervals, and after four exposures the current was reversed. It is of great interest that the reversal brought the boundaries accurately back to their original positions.

Some of the characteristic features of the schlieren method may be considered with the help of figure 4. The angular deviation of a pencil of light in the boundary is proportional, under appropriate conditions, to

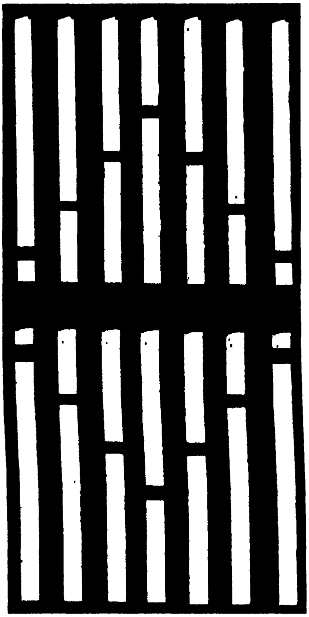

FIG. 5. Schlieren bands of a single protein. Exposures were made at 30-min. intervals and the current was reversed after the fourth exposure.

the gradient, dn/dx, of the refractive index, n, and the horizontal breadth, a, of the boundary. The displacement, Δ, of the schlieren diaphragm necessary to intercept the deflected pencil is also proportional to the "optical lever arm," b. We therefore have the expression

$$\Delta = ab \frac{dn}{dx} \tag{1}$$

in which a and b are constants of the apparatus, and dn/dx varies vertically through the boundary. As the schlieren diaphragm is raised, the first pencils of light to be intercepted are those which have passed through the steepest gradients of refractive index, i.e., the center of the boundary if the diffusion and spreading of the latter have been normal. Thus a series of photographs of the boundary with decreasing displacement of the diaphragm give an indication of the variation of the refractive index through the boundary. Such a series of photographs of the boundaries formed in a mixture of rabbit and guinea pig hemoglobins is shown in figure 6, the upper half of the figure referring to rising boundaries and the lower to descending boundaries.

Since the refraction of the solution is substantially proportional to the concentration of protein, the schlieren method may be used to obtain quantitative estimates of the concentrations of the various constituents. Thus, if the cell image at the photographic plate is masked by a narrow vertical slit, a slow horizontal movement of the plate simultaneously with the vertical displacement of the schlieren diaphragm produces automatically on the plate a transparent area proportional to $\int \Delta \, dx$. Reference to equation 1 shows that

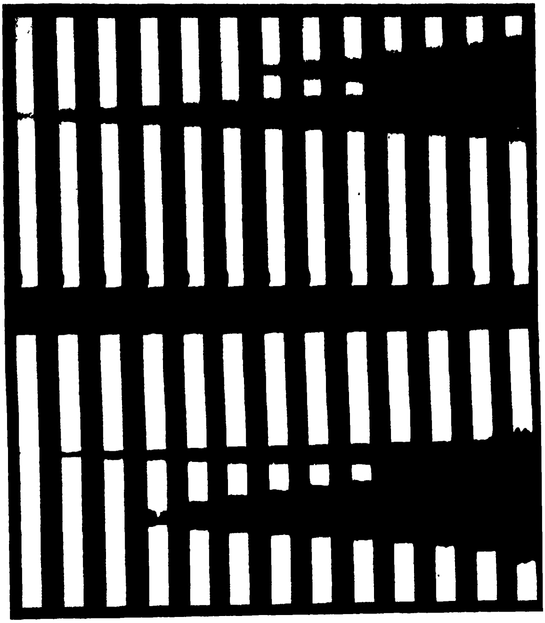

FIG. 6. Schlieren bands, for different schlieren diaphragm settings, of a mixture of rabbit and guinea pig hemoglobins.

$$\int \Delta \, dx = \int ab \frac{dn}{dx} \, dx = ab\Delta n$$

in which Δn is the refractive index increment due to the protein constituent causing the boundary in question. This modification of the schlieren method for quantitative analysis has been described in two papers from this laboratory (6, 7).

REDUCTION OF THERMAL CONVECTION

The chief problem in electrophoretic methods is the elimination of mixing due to thermal convection. If a current of electricity is passing through a conducting solution in a tube, heat is generated in each volume element of the solution but flows to the thermostat only through the wall of the tube. The solution along the axis of the tube is thus hotter than at the wall. Normally, therefore, the solution at the wall will be heavier, and in falling will give rise to convection currents. We shall consider, as a typical example, a current of 0.006 ampere passing through a solution the specific conductance of which is 0.0038 mhos (0.1 N sodium acetate at 0°C.) in a cylindrical tube of 5 mm. internal and 10 mm. external diameter. The formula[2] which describes the temperature of the solution, t_s, in the steady state as a function of the distance, r, from the axis is

$$t_{r=0} - t_s = \frac{I^2 \epsilon}{4\kappa K_s} r^2 \tag{2}$$

The corresponding formula for the temperature of the glass, t_g, is

$$t_g - t_0 = \frac{a^2 I^2 \epsilon}{2\kappa K_g} \ln \frac{b}{r} \tag{3}$$

In these equations a and b are the inside and outside radii, respectively, of the tube, K_s and K_g are the thermal conductivities of the solution and glass, I is the current density, ϵ is the electrical equivalent of heat, and t_0 is the thermostat temperature. Using equation 2, the solution along the axis of the tube is 0.65°C. hotter than at the wall, and from equation 3 the drop in the wall is 0.67°C. The computed temperature distribution is given in figure 7a, in which the temperature increase, Δt, over that of the thermostat is plotted against the distance from the axis of the tube. If the thermostat is regulating at 25°C., this temperature gradient in the buffer solution is accompanied by the density variations shown in figure 7b and, as has been stated, it is these differences which cause mixing, by convection currents, of the solution in the tube. If, on the other hand, the thermostat is regulating at 0°C., the density differences in the solution are much less and the variation is in the opposite direction, as shown in figure 7c. The contrast between the curves of 7b and 7c arises from the fact that this buffer solution has a maximum density at 2.85°C. (2). If, in this particular example, the thermostat temperature were regulated at 1.85°C., the average temperature in the tube would be 2.85°C., and the density gradient would be a minimum, as indicated by the horizontal line in figure 7d. Some preliminary measurements of the temperature variations in a rectangular channel indicate that they are of the same order of magnitude as those indicated in the example just given for a cylindrical tube.

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[2] These equations are from a private communication from Dr. Melvin Mooney. The authors wish to acknowledge gratefully this aid, which has been of service to them both in this work and in their earlier work on the determination of transference numbers by the moving boundary method.

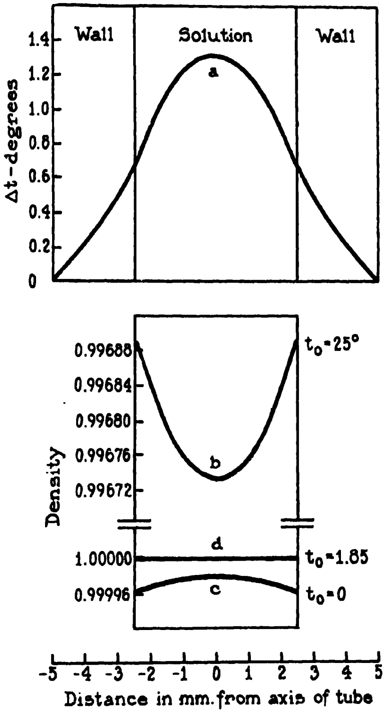

FIG. 7. Distribution of temperature and density in a salt solution, in a cylindrical tube, during passage of electric current.

In spite of the fact that the density differences produced by temperature gradients are, as indicated in figure 7, very small, the recent great advances in the electrophoretic method are due to the important observation of

Tiselius that convection can be largely eliminated by working at temperatures near that of maximum density. The computations suggest that further progress may be made by additional knowledge of the temperature of maximum density of the buffer solutions used and of the actual temperature distribution in the electrophoresis cell. Investigations along both these lines are in progress by Dr. T. Shedlovsky of this laboratory.

SOME TYPICAL APPLICATIONS OF THE ELECTROPHORETIC METHOD

One of the most interesting uses of this electrophoretic method is the determination of the mobilities of proteins as a function of the pH and the ionic strength of the buffer solution in which they are dissolved. The following data for a typical experiment were obtained in collaboration with Drs. Karl Landsteiner and van der Scheer (4). The protein was crystalline albumin from guinea hen eggs. A 0.5 per cent solution was prepared in a 0.02 normal acetate buffer at pH 5.19. The specific conductances, at 0°C., of the buffer and protein solutions, as measured on the bridge described by Shedlovsky (10), were essentially the same and equal to 0.000832 mho. The current, measured potentiometrically, was practically constant throughout the experiment and equal to 0.00679 ampere. From a calibration with mercury the cross-sectional area of the channel in the cell was known to be uniform and equal to 0.815 sq.cm. The current density was, therefore, 0.00833 ampere and the electric field 10.01 volts per centimeter. The schlieren bands were photographed, using unit magnification, at 30-min. intervals and are shown in figure 5. As mentioned earlier in connection with this figure, the current was reversed after the fourth exposure and the boundaries returned accurately to their original position. The displacement of the boundary moving upward into buffer during the 90-min. interval before reversal of the current was 2.05_5 cm., giving $3.80_5 \times 10^{-4}$ cm. per second as the velocity and -3.80×10^{-5} cm. per second per volt per centimeter as the mobility of the protein, the migration being anodic. The corresponding displacement of the boundary moving into protein was 1.99_5 cm., giving a mobility of -3.70×10^{-5}. The average from the two boundaries is thus -3.75×10^{-5}. The greater displacement of the boundary moving into buffer has been quite generally observed and will be discussed later in this paper.

In addition to the measurement of mobilities the method furnishes information as to the purity and homogeneity of the protein solution and also may be adapted to separation of the components of a mixture. This latter application, called "electrophoretic analysis" by Tiselius, is illustrated in figure 8. Suppose a mixture of proteins A, B, and C, for which the (positive) mobilities are $u_A > u_B > u_C$, is placed in the cell as shown in figure 8a. Movement of the bottom center section of the cell to the right will bring the protein and buffer solutions into contact in the plane α. On passage of a current three boundaries will appear in the upper cathode and in the lower anode sections, as is shown in figure 8b. It is evident from the figure that, in the ideal case assumed, there is a separation of pure component A in the region between the two leading boundaries on the cathode side and of pure component C between the two slowest boundaries on the anode side. However, if the electrolysis is continued as indicated, before any large proportions of A and C have been separated, the boundaries will have migrated out of the cell in one case and into the bottom section in the other. However, if the second boundary could be given an apparent velocity of zero, as indicated in figure 8c, while the leading boundary moves through the length of one section, isolation of this section from the others would make possible the recovery of a solution of pure A from the upper cathode portion of the cell. Simultaneously, as indicated, the slowest component would have an apparent negative velocity, and pure C could be recovered from the upper anode section. As a matter of fact, a boundary can be given any apparent velocity desired by a displacement of the entire solution of the cell. Such displacements have been accomplished in a variety of ways. In Smith's (11) moving boundary apparatus this was done by the withdrawal of mercury, while Collie and Hartley (1) used a piston driven by clockwork. In Tiselius' apparatus he displaces the boundaries by withdrawing, with clockwork, a loosely fitting plunger in one of the electrode vessels. We have used this method also, but in the more recent apparatus described in this paper we have

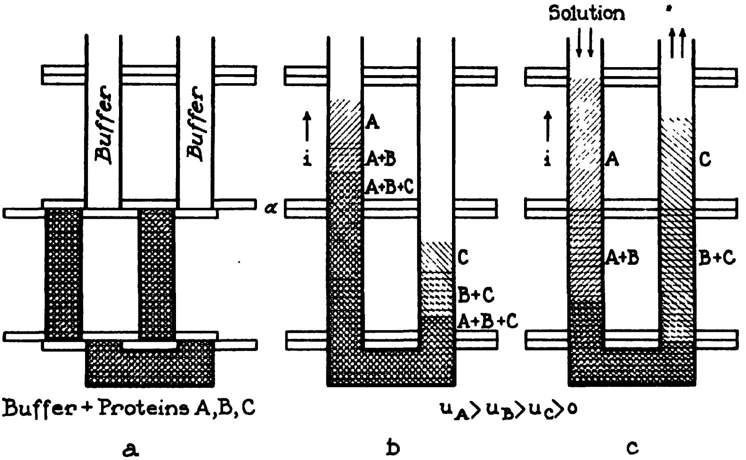

FIG. 8. Ideal electrophoresis of a protein mixture

kept one electrode vessel closed and have displaced the solution in the cell by forcing buffer into this side from a syringe, the piston of which is displaced, at the desired rate, by a threaded rod operated by a synchronous clock motor.

Figure 9 is the electrophoretic pattern, obtained in collaboration with Dr. Karl Landsteiner, of a water-insoluble fraction of a naturally occurring mixture of plant proteins. The three bands indicate the presence of three well-defined constituents. Having demonstrated the complexity of the protein material, it was of importance to locate a certain biological activity which one of the components possessed. To this end the leading components were separated, using the compensation method outlined above. They exhibited no activity, whereas the slow component did possess the activity. An electrophoretic study of the water-soluble fraction, which was also active, indicated the presence of a single protein with a mobility near that of the active material in the water-insoluble fraction. The possibility remained that the two active proteins were identical, the ammonium sulfate separation having possibly been incomplete. This question was answered unambiguously by analyzing electrophoretically a mixture of the two active proteins. Two bands were obtained, indicating the separate identity of the two proteins.

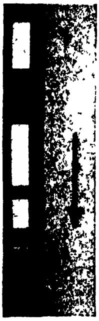

FIG. 9

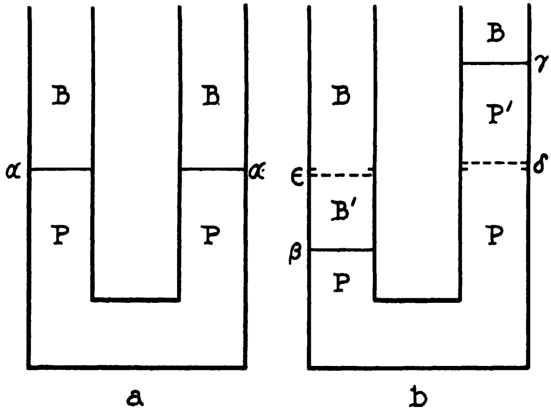

FIG. 10

FIG. 9. Electrophoretic pattern of water-insoluble fraction of a mixture of plant proteins.

FIG. 10. Diagrammatic representation of boundary formation and migration during electrophoresis.

In electrophoretic analysis, using compensation, it is frequently necessary to pass relatively heavy currents through the cell for long periods of time. Under these conditions either very large electrode vessels must be used, or a layer of concentrated buffer solution must be introduced (17) between the concentrated chloride solution around the electrode and the dilute buffer solution above. Tiselius (15) has given an excellent discussion of the size of electrode vessel required when no concentrated buffer solution is used.

BOUNDARY ANOMALIES

An electrophoresis experiment with a single protein may be represented diagrammatically by figure 10. Initially boundaries are formed at the level α in the two sides of the cell between the buffer solution at a concentration B and the buffer solution of protein at a concentration P (in which the protein is assumed to be negatively charged). Passage of a current causes one of these boundaries to descend through a volume V_P to a new position β (figure 10b), and the other to rise through a volume V_B to the position γ. Under ideal conditions, realized closely with a dilute solution of a homogeneous protein in a buffer of sufficient strength to control the pH and conductance of the system, the volumes V_P and V_B will be equal. With more concentrated protein solutions, however, so-called "boundary anomalies" are observed. These are illustrated in figures 11 to 13. For instance, the schlieren bands shown in figure 11a for different settings of the schlieren diaphragm indicate a boundary that has risen into buffer and 11b the boundary that has descended into the protein solution in the other side of the cell. It is quite evident that the boundary moving into buffer is the sharper of the two. This is quite generally observed. A more disturbing anomaly is shown in figure 12, in which the relative displacement of the schlieren bands indicates that the rising boundary is moving more rapidly than the descending one; this is also, apparently, a general rule. Another anomaly is illustrated in figure 13. In this experiment with a 2.5 per cent solution of a single protein the boundaries were formed in the usual manner. They were then shifted in the cell with the compensation device to the positions shown in column 1, so that a very slowly moving boundary, which might normally be obscured by the horizontal glass plates, could become visible. On passage of a current the protein boundaries migrated as usual to the new positions indicated in column 2 of the figure. However, on raising the schlieren diaphragm a second, and relatively faint, boundary, δ (column 3), appeared in the protein solution but slightly removed from the original position of the boundary. In the discussion to follow it will become evident that this is not due to a second protein, but arises from a gradient of concentration left behind by the advancing protein boundary. It is similar to the "δ globulin" boundary of Tiselius (16, 13). On raising the diaphragm still further, at a somewhat later time, a very faint boundary appeared in the buffer solution at ϵ (column 4) and was similar in nature to the δ-boundary.

In practice the protein solution is prepared by dialysis against the buffer solution. It is important to recall that a difference of buffer salt concentration, between the two solutions, exists when the dialysis is complete, owing to the Donnan equilibrium. When passage of a current causes a boundary between two such solutions to move (from α to β, figure 10) there is formed in the intervening volume, V_P, a buffer solution of composition B'., This composition has been "adjusted," in general, to a value different from B in such a way that its "regulating function" has the same value as that of the protein solution it has replaced. To quote an earlier

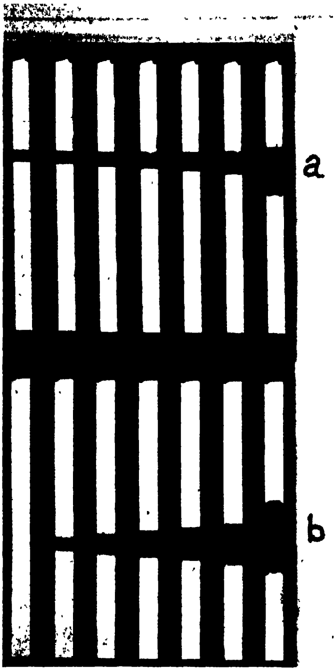

FIG. 11

FIG. 12

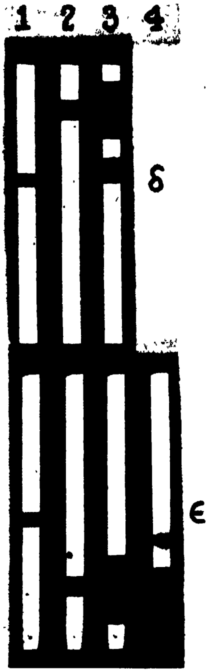

FIG. 13

FIG. 11. Schlieren bands, for different diaphragm settings, of a single protein. The boundary (a) moving into buffer is sharper than that (b) moving into protein.

FIG. 12. Schlieren bands of a single protein. The velocity of the boundary moving into buffer is greater than that of the boundary moving into protein.

FIG. 13. Schlieren bands of a single protein. The δ and ϵ bands are due to different concentrations of the same substance.

paper by the authors (8), "This (regulating) function defines a property of the solution which, at any given point, retains a constant value independent of changes of concentration caused by electrolytic migration. If, as a result of such migration, species of ions different from those initially present appear at a point, their concentrations will be adjusted to values compatible with the constant determined by the *initial* composition of the solution." It may be noted here that the concentration changes which occur behind the boundary moving into the protein solution are similar to those encountered by one of the authors (5) in the electrolysis of mixtures of hydrogen and potassium chlorides. The boundary ϵ thus forms between two solutions of the same salt, but at the different concentrations *B* and *B′*. It is stable if the lower solution is the more concentrated. Boundaries of this type have been investigated theoretically by Kohlrausch (3) and Weber (18) and experimentally by Smith (12) and ourselves. That such boundaries persist and move on the passage of a current is illustrated in figure 14. Boundaries were formed between 0.01 and 0.1 normal solutions of lithium chloride; these were shifted from behind

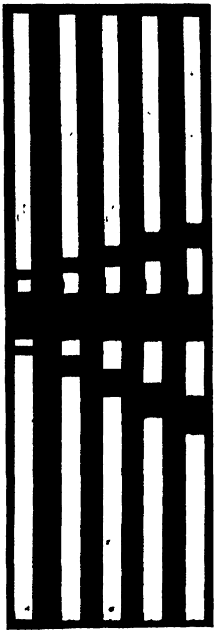

FIG. 14. Schlieren bands of the concentration boundaries between 0.01 and 0.1 normal solutions of lithium chloride.

the horizontal plates and current was passed. The motion of the boundaries, actually very slow compared with others dealt with in this paper, is indicated by the displacement of the schlieren bands. The motion is due to a change of the transference numbers with concentration.

With the boundary moving into buffer there is a similar but more complicated adjustment of the composition of the protein solution which replaces the buffer as the boundary rises. The resulting concentration boundary, δ (figure 10), between the solutions P and P' moves slowly under the influence of the current and, as mentioned above, is similar to the "δ globulin" boundary of Tiselius. The greater visibility of this boundary, compared with the ϵ-boundary, may be due to the fact that the first involves a gradient of protein concentration whereas the second does not.

In order to account for the boundary anomaly illustrated in figure 11, i.e., the greater diffuseness of the boundary moving into the protein solution, we must consider the effect of the concentration changes, discussed in the preceding paragraphs, upon the specific conductances of the solutions. Owing mainly to its relatively high viscosity, the specific conductance, κ_P, of the protein solution is generally lower than that, κ_B, of the buffer. The conductance differences at the actual boundaries, β and γ of figure 10b, are further increased by the fact that $\kappa_{B'} > \kappa_B$ and $\kappa_{P'} < \kappa_P$. Consequently for a given current the electric field is greater in the protein solution than in the buffer, and variations of the field exist at the boundaries. The dilute uppermost layers of the boundary β moving into the protein solution thus find themselves in weaker fields than do the more concentrated layers and thus tend to lag behind, causing the boundary to become diffuse. In the case of the boundary γ moving into buffer, however, the dilute, slowly moving layers tend to be overtaken by the faster, concentrated ones, with the result that the boundary tends to become sharper. (See also reference 14, page 28.)

If there is a stable δ-boundary, as shown in figure 13, the total concentration below the boundary must be greater than above it. Moreover, it was found in an actual case that the solution P' had a lower conductance than the solution P. The potential gradient above the δ-boundary will therefore, in general, be greater than below, with the result that the protein must move more rapidly in that region than in the main body of the solution. If the displacement of the boundary γ can be assumed to be a measure of the velocity of the protein particles in the solution P', this boundary would be expected to move more rapidly than the β-boundary, as has been true in every case we have observed.

In the preceding discussion of boundary anomalies we have ignored the small differences of pH, required by the Donnan equilibrium, which exist between the buffer and protein solutions. Owing to the change of protein mobility with pH, these differences at the boundaries can cause anomalies similar to those mentioned. Both theory and experiment indicate, however, that by far the major portion of these anomalies arises from the differences in specific conductance.

Although boundary anomalies are always present to some extent they may be reduced by proper selection of the buffer solution. The buffer used as solvent for the protein should have a high capacity in order to reduce, relatively, the buffer action of the protein itself. It should also have a low specific conductance in order to decrease disturbances due to the heating effect of the current. Since both buffer capacity and conductance increase with the concentration of buffer salts, it is evident that these two conditions are mutually incompatible, and a compromise must be made.. As buffer capacity does not depend upon ionic mobilities, buffer salts the ions of which have low mobilities should be selected if possible. We have used sodium salts in preference to potassium salts for this reason. Lithium salts would be still better, if circumstances justified their preparation. High concentrations of weak electrolytes should be avoided in the preparation of buffers, unless allowance is made in the thermostat temperature for the large effect of these components on the temperature of maximum density of the solution.

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THE EFFECT OF CERTAIN TISSUE EXTRACTS, OF AMMONIA SALTS AND OF CERTAIN AMIDES ON THE RATE OF FERMENTATION BY BAKERS' YEAST

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(October 20, 1938)

If cells of bakers' yeast are suspended in phosphate buffers or in sodium or potassium dihydrogen phosphate under anaerobic conditions they ferment added glucose at a very rapid rate. This is, however, not usually the optimal rate of fermentation and a stimulation of this rate has frequently been described.

The process of fermentation must be considered as a cycle of interdependent reactions. The rate at which the complete cycle occurs is governed by the rate of the slowest reaction. Depending upon the conditions of the experiment and the previous history of the yeast cells any one of a number of different steps may be the rate controlling step. Presumably the addition in available form of whatever factor is limiting the rate will stimulate fermentation and the addition of any factor already present in excess, no matter how essential this factor may be, will not stimulate fermentation. It is not necessary that the stimulating factor participate directly in the cycle of reactions, it may "activate" a participating factor or may exert an effect on the permeability of the cell membrane. From such considerations it is not to be expected that stimulation of fermentation is due to a specific factor except under specific conditions. It is therefore not surprizing that a number of substances have been described as stimulating fermentation.

I have recently had occasion to observe that if an aqueous extract of any one of a number of animal organs is added to the cells the rate of fermentation is increased. Such observations have been made before and in a series of papers from EULER's[1]) laboratory this effect of extracts (chiefly yeast extracts) has been studied. The stimulating effect has been attributed to a definite factor (or possibly two factors) to which they have given the name "*activator Z.*" This factor is clearly different from cozymase (2).

The existence of some such factor has been confirmed by the work of MEYERHOF and IWASAKI[3]), BORCHARDT and PRINGSHEIM[4]), and NORRIS and KREKE[5]). The factor has not been identified. Its relationship to the various growth factors is discussed in references ([1]) and ([5]). SCHULTZ, ATKINS, and FREY[6]) have recently reported that thiamin stimulates fermentation and have recommended this action as a means of testing for this vitamin. It seemed possible that thiamin might be the factor Z. However, in the work described below tissue extracts activated fermentation and thiamin did not. This is not considered as contradictory to the work of SCHULTZ, ATKIN and FREY, but merely as evidence that thiamin is not the limiting factor under the conditions tested here. Similarly, LAZINTSKI and SZÖRENYI[7]) obtained stimulation of fermentation with yeast cells by the addition of potassium chloride, but under the conditions used here potassium chloride gives no stimulation. The experiments described below were carried out in an effort to see what factors in these extracts were causing this stimulation.

EXPERIMENTAL

The yeast used in most of these experiments was FLEISHMANN's bakers' yeast obtained in small cakes from grocery stores and washed as previously described ([8]). The experiments were carried out with the usual WARBURG manometric apparatus at 28° C. The test solutions were mixed with secondary phosphate and the vessel then filled with 100% CO_2. The final ph was thus that of a CO_2 saturated primary phosphate solution.

Figure 1 shows the effect on fermentation of a preparation made by extracting fresh bull testicle with 1,5 parts of 0,1 N acetic acid. Such crude extracts permit a multiplication of the yeast cells. In a two hour experiment the number of cells may increase as much as 55%. Such multiplication can usually be seen from the curve of CO_2 production per minute as is illustrated in figure 2. The continuing rising curve indicates reproduction. Purified preparations retain their power to stimulate fermentation without permitting multiplication of the cells. The effect of two fractions of the crude extract is also shown in figure 1.

An attempt was made to isolate the factor responsible for this stimulation. After a rather long and somewhat tedious procedure a well crystallized active product was obtained. This substance proved to be *ammonium chloride*.

If "reagent" ammonium chloride is purified by sublimation and then added to the yeast cells it has the stimulating effect shown in figure 3. This increase in fermentation is not accompanied by an increase in the number of cells as shown in figure 2.

The effect on oxygen consumption is to increase it very slightly. This is not shown in the figure. It may be observed (figure 3) that the effect on aerobic fermentation is of the same order as the anaerobic effect. Other ammonium salts have essentially the same effect. The actual percentage increase in CO_2 formed depends on the yeast used. With a brewers' yeast tested it was very small, with a torula yeast it was only moderate and with bakers' yeast washed three times in distilled water it was of the order of 50% increase for a two hour experiment. The effect of the same amount of ammonium chloride can be increased to about 100% stimulation by first shaking the cells in a glucose salt solution for two hours,* then centrifuging them out and washing them before the test. If the cells are treated in the same way in the absence of glucose the increased sensitivity to ammonium salts does not develop.

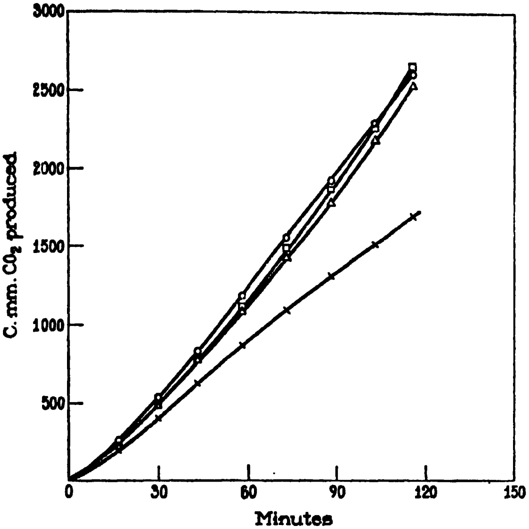

FIGURE 1. Effect of tissue extracts on fermentation.

× control (4 mg. dry weight of yeast cells in NaH_2PO_4. Glucose is 3%).

○ same as control + 0,5 cc. testicle extract.

△ same as control + 0,1 cc. acetone precipitate of testicle extract dried, extracted with 10 parts H_2O and dialyzed.

□ same as control + 0,5 cc. acetone filtrate of testicle extract, after removal of acetone.

It was observed by Euler and Cassel (9) in 1913 that the addition of ammonium formate to fermenting yeast cells definitely accelerated the rate of fermentation. They attributed the effect to the formate ion rather than the ammonium ion. In 1934 Euler and Larsson (1) observed that if a certain preparation of activator Z was neutralized with ammonium hydroxide it was quite active, but if neutralized with $Ba(OH)_2$ it was much less active. They considered that the difference in activity might be due to an accelerating effect of ammonia so they tested the effect of ammonium sulfate but apparently found no comparable effect. They remark that the result is difficult to understand. It is made more difficult to understand by the fact that Zeller (10) in 1926 had found under similar conditions that a series of ammonium salts did rather markedly stimulate fermentation.

The question now arises whether the effect of the testicle extract is due to ammonium salts contained in it or whether the two effects are merely similar. To answer this the following experiments have been carried out.

A distilled water extract of a dried acetone precipitate of testicle extract stimulates fermentation. Dialysis in cellophane for forty-eight hours against flowing distilled water does not remove the stimulating factor (curve Δ figure 1). Trichloracetic acid precipitates the active factor. Trypsin does not destroy the factor but renders it diffusible and non-precipitable by trichloracetic acid. Heating at 110° for 20 hours with strong sulfuric acid (1:3) does not destroy the stimulating factor but renders it diffusible.

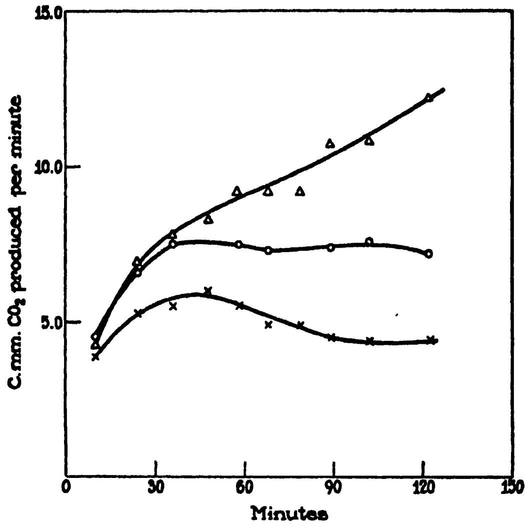

FIGURE 2. Curves of CO_2 production per minute.

× control (2,5 mg. dry weight of cells in M/15 NaH_2PO_4. Glucose is 3%).

○ same as control + 0,4 mg. NH_4Cl.

Δ same as control + 0,5 cc. testicle extract (the continued rising curve indicates reproduction of cells).

At the end of the experiment the contents of each vessel were diluted to 25,0 cc. and the cells counted. The control contained 3220 per cmm, the NH_4Cl solution 3320 per cmm, and the testicle extract 5,000 per cmm.

The filtrate from the acetone precipitation of the testicle extract contains a fermentation stimulating factor. This is diffusible. It gives no ammonia reaction with NESSLER's reagent under conditions where an added ammonium salt reacts well. The active factor does not distill from an alkaline solution under diminished pressure at 50° C. The distillate received in 0,1 N hydrochloric acid contains practically no active factor and practically no base. Refluxing the alkaline solution for one hour results in the formation of a volatile active factor. Titration of the hydrochloric acid used to receive the distillate from the refluxed solution shows that it has been to a large extent neutralized and this solution gives an intense reaction with NESSLER's reagent. Refluxing the solution with acid also forms a volatile active factor. The solutions remaining after distilling off one half the original volume are proportionately less active. Similarly, if the solution of the non-diffusible factor is made 1,0 M. with sodium hydroxide and refluxed for one hour a volatile and diffusible active factor which reacts with NESSLER's reagent is formed. The protein, or protein-like substance that remains after dialysis retains only very little of its original activity.

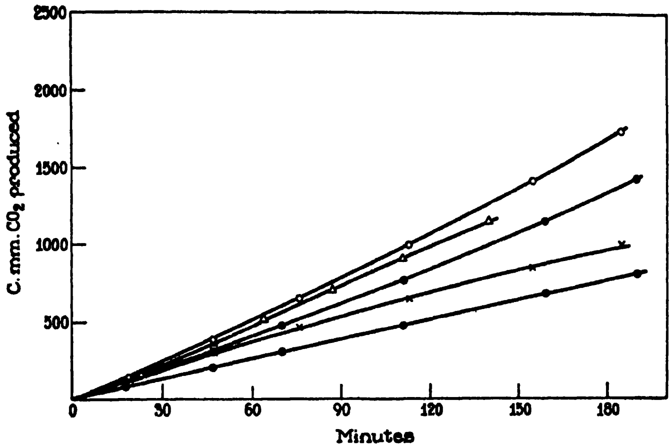

FIGURE 3. Effect of ammonium-chloride on fermentation.

× anaerobic control (2,7 mg dry weight of cells in M/15 NaH_2PO_4. Glucose is 3%).

O same as anaerobic control + 0,4 mg NH_4Cl.

△ same as anaerobic control + 0,2 mg NH_4Cl.

⊗ aerobic control (total CO_2 production).

● same as aerobic control + 0.4 mg. NH_4Cl.

The above results show clearly that the activity of the testicle extract is present in at least two forms neither of which is ammonia.

Heating either the diffusible or the non-diffusible form with acid or alkali forms ammonia and destroys the non-ammonia factor. If we assume that the ammonia formed is formed from the active compounds then amino acid amides which can exist free, as part of low molecular weight peptides, or as part of proteins possess the properties necessary to explain the results. The activity of *glutamine**) and *asparagine* has accordingly been tested and the results are shown in figure 4. It may be seen that both actively stimulate fermentation.

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*) The glutamine was very kindly supplied by Dr. H. B. VICKERY.

The only substance in addition to those mentioned that was found to possess some significant activity was arginine. It is considerably less active than an equimolecular amount of ammonium chloride, but still definitely active. The product used was Hoffmann LaRoche's *d*-arginine. Its solution gave no test for ammonia. The reason for its activity is not clear since, as stated below, ornithine, urea and guanidine are all inactive.

The following substances have been tested and found to possess no significant stimulating action under our conditions: acetamide, nicotinic acid amide, allantoin, urea, thiourea, cozymase, riboflavin, thiamine, magnesium sulphate, manganese sulphate, potassium nitrate, potassium chloride, calcium chloride, creatine, creatinine, guanidine, glycine, ornithine, histidine, aspartic acid, glutamic acid, cysteine, glutathione, gelatine, casein, egg albumin, monomethylamine, dimethylamine, trimethylamine, monoethylamine and hydroxylamine. Of this group only hydroxylamine showed strong inhibition although a number of the others inhibited slightly.

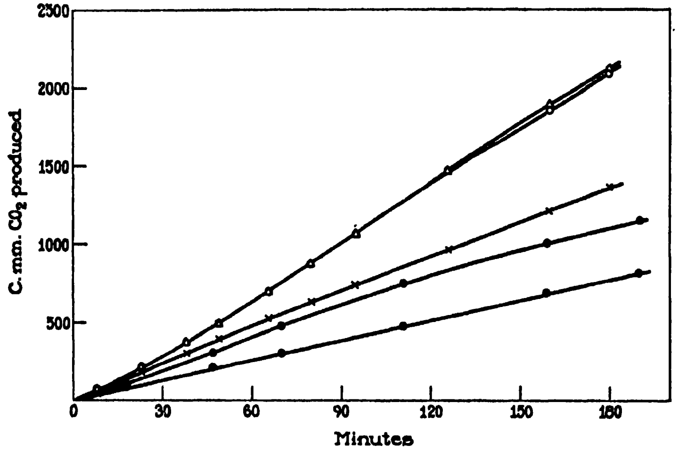

Figure 4. Effect of asparagine and glutamine on fermentation.

× anaerobic control.

△ same as anaerobic control + 1,1 mg asparagine.

○ same as anaerobic control + 1,1 mg glutamine.

⊗ aerobic control (total CO produced).

● same as aerobic control + 1,1 mg glutamine.

The above results do not indicate whether the glutamine and asparagine owe their activity to the fact that they form ammonia or whether the ammonia is active because it is converted into glutamine or asparagine or whether the two act independently. The fact that the effects of the two are not additive if either is added in excess indicates that they do not act independently, although this is not sufficient proof. The effect of these reagents on a cell-free extract prepared from bakers' yeast with M/15 Na_2HPO_4 as described by LIPMANN[11]) indicates that the *active substance is the ammonia.*

This is demonstrated in figure V. Here the ammonium chloride exerts a striking effect on shortening the induction period and the two amides have much less effect. The curves shown were obtained under aerobic conditions. A similar

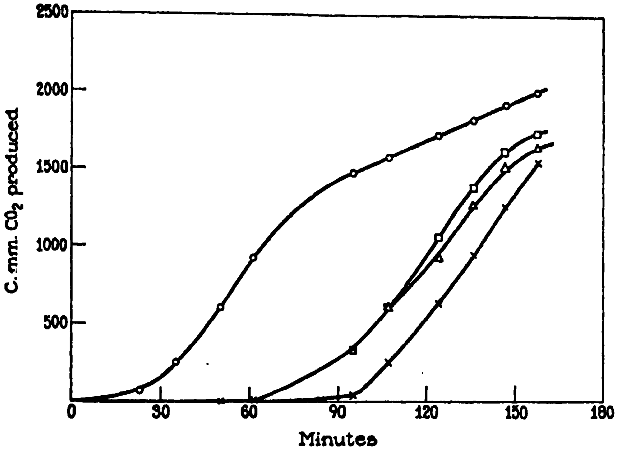

FIGURE 5. Effect of NH_4Cl, asparagine and glutamine on a 3 hr. old Na_2HPO_4 extract of dried bakers' yeast.

× control (0,6 cc. extract + 0,7 cc. H_2O + 0,1 cc. 40% glucose. Air in gas room).

O same as control + 0,4 mg NH_4Cl.

△ same as control + 1,1 mg asparagine.

□ same as control + 1,1 mg glutamine.

but less profound effect is obtained under anaerobic conditions. If the extract is used immediately after preparation the induction period of the control is shorter and the effect of the ammonium chloride is accordingly less but it is still very definite. It is well known that hexosediphosphate will shorten the induction period of a yeast extract, but, so far as I am aware, this is the first demonstration that an ammonium salt can have a similar effect on an extract. This gives a definite clue to the action of the ammonia. The induction period of an extract is due to the difficulty that it has in starting the process of phosphorylating the glucose. Hexosediphosphate accelerates this process because it is itself fermented and its phosphate is eventually transferred to more glucose. To produce a similar result ammonia must accelerate the esterification of inorganic phosphate. It is believed that the inorganic phosphate is first combined with the adenylic acid system (12). Adenylic acid contains a free amino group and an enzyme is known that splits off this amino group forming inosinic acid and ammonia. This is believed to be the source of the ammonia formed in muscle contraction (13). Inosinic acid is much less active or inactive in transporting phosphate. The addition of ammonium chloride to an extract would tend to favor the formation of adenylic acid from inosinic acid and this transformation may be connected with the phosphorylation of the adenylic acid and eventually of the glucose.

The above data suggest that in cases where the fermentation stimulating action of extracts is being considered it would be well to consider that part of the effect may be due to ammonia or to some precursor of ammonia such as glutamine or asparagine.

SUMMARY

The stimulating effect of a testicle extract on fermentation by cells of bakers' yeast is described. An attempt to isolate the active principle led to the isolation of ammonium-chloride. It is shown that sublimed ammonium-chloride in amounts as low as 0,2 mg per WARBURG vessel may increase fermentation as much as 100%. Asparagine and glutamine exert a stimulating effect on yeast cells very similar to that of ammonium salts. Ammonium salts markedly reduce the induction period of a cell-free extract prepared from bakers' yeast. Asparagine and glutamine have much less effect on such a cell-free extract.

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ARTIFICIAL MAINTENANCE MEDIA FOR CELL AND ORGAN CULTIVATION

I. The Cultivation of Fibroblasts in Artificial and Serumless Media*

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(From the Laboratories of The Rockefeller Institute for Medical Research)

Plate 19

(Received for publication, November 23, 1938)

The importance of developing artificial media that can be used in the place of serum for maintaining the life of tissues and organs outside the body hardly needs to be emphasized. Many of the studies for which the organ culture technique[1] was developed, as well as others that can be carried on by the simpler methods of tissue culture, depend for their success on the creation of suitable artificial media. These media are needed to reduce the cost of experimentation, to make possible extensive cultivation of human organs and those of small animals from which serum in sufficient quantity cannot be obtained, and for all studies in which the production of serum and other protein substances is to be investigated. For the latter purpose, media that are serumless and free from protein will be required. But for other work media that contain serum as one constituent may be used.

Several artificial media designed to promote rapid growth of cells in tissue culture have already been described.[2,3] But these are not suitable for studies in which the functioning of organs and tissues is to be investigated. For such studies media that will maintain cells without promoting growth are required. The purpose of this report is to describe media devised for this purpose, and the results obtained when they were used to sustain the life of a pure strain of fibroblasts *in vitro*. Experiments in which they were used for organ cultivation will be described in another communication.[4]

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* Reported in brief in *Proc. Soc. Exp. Biol. and Med.*, 1938, **39**, 291.

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Composition of the Media

In the course of this work many different media with varying combinations of constituents have been used. For the sake of brevity only four will be described. Their composition will be given first, and then a description of the way in which they can be prepared.

Medium I.—

Whole blood digest, as described further on, in amount to give 30 to 60 mg. per cent nitrogen

Serum	2 or 3	per cent
Phenol red	5 mg.	" "
Tyrode's solution		

Medium II.—

Whole blood digest to give either 30 or 60 mg. per cent nitrogen

	per 100 cc.	
Cysteine hydrochloride	9.0	mg.
Insulin	0.1	unit
Thyroxine	0.001	mg.
Hemin	0.004	mg.
Vitamin A (containing some D)[5] dissolved in serum	100.0	units
Vitamin B_1	0.1	gamma
Vitamin B_2	3.4	gammas
Ascorbic acid	0.3	mg.
Glutathione	1.2	mg.
Glucose	200 to 300	mg.
Phenol red	5.0	mg.
Potassium iodide	0.13	mg.
Salts as in Tyrode's solution		

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[4] Some experiments in which organs have been cultivated in these media have already been described by Carrel, A., and Lindbergh, C. A., The culture of organs, New York, Paul Hoeber, Inc., 1938.

[5] The vitamin A was prepared from haliver oil and contained 1 unit vitamin D for each 5 units of vitamin A.

Many of the constituents used in this medium were selected because they had previously been found either by Vogelaar and Erlichman[3] or by Baker[2] to prolong the life of cells in artificial, growth-promoting media. The concentrations of the individual constituents have been adjusted to those that seemed best suited to maintenance.

To bring the vitamin A into a form in which it could be taken up by the cells, it was dissolved at high concentration in serum. Then a small amount of this serum, about 0.07 per cent, was used in the medium.

Medium III.[6]—This contained all the constituents listed under medium II and in addition:

	per 100 cc.	
Tryptophane	5 to 10	mg.
Witte's peptone to give	6.0	mg. nitrogen
Sodium glycerophosphate	57.5	mg.
Urea	2.4	mg.
Glycerine	0.2	cc.
Thymus nucleic acid[7]	20.0	mg.
Antuitrin	0.2	cc.
Adrenalin chloride[8] (1:1000)	0.1	cc.
Eschatin (suprarenal[8] cortex hormone)	0.1	cc.
Pitressin[8] (pituitary hormone)	0.1	cc.

Medium IV.—This contained all the constituents used in medium III with the exception of the vitamin A. It was, therefore, a serumless medium.

Preparation of the Blood Digest.—Approximately 700 cc. of cow blood is obtained from the slaughter house. Coagulation is prevented during delivery by having the blood collected in a bottle containing 50 mg. of heparin[9] dissolved in 20 cc. of Ringer's solution. 450 cc. of this blood is shaken with 225 cc.[10] of chloroform and placed in an incubator at 37°C. overnight. The next day the solidified mass is broken up, either by macerating it in a mortar, or passing it through a meat chopper. It is then diluted with distilled water to a volume of 6 liters. N/1 sodium hydroxide solution is added in quantity sufficient to bring the pH of the mixture as measured by the glass electrode to 10.2.[11] Then 10 gm. of Armour's pancreatin is added and the mixture is incubated for 24 hours, being shaken at half hour intervals during the first few hours. The next day, alkali is again added to bring the pH to 8.3. Then 4 gm. more pancreatin is added and digestion is continued without further adjustment of pH for 2 days. After this, the mixture is filtered through glass wool, and then through filter paper. The volume of the filtrate is measured, and trichloracetic acid is added in quantity sufficient to make its concentration 2.5 per cent. After standing at room temperature for 16 hours, the clear, supernatant fluid is siphoned off, and the remaining cloudy fluid centrifuged. The siphoned fluid and that obtained on centrifuging are combined and boiled in an open basin to approximately half their original volume. This destroys the enzyme, decomposes the trichloracetic acid, and removes the chloroform. The cryoscopic point is determined and the fluid made isotonic by addition of salt or water as required. Total and amino nitrogen determinations are made and the fluid is sterilized by autoclaving. The ratio of amino to total nitrogen of digests so prepared varies from 0.42 to 0.45. Samples of the digest saturated with ammonium sulfate show only the faintest trace of precipitate.

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[6] Though many experiments have been made in the course of this work, with the individual constituents, to ascertain the nature of the substances needed and the concentrations at which each should be used, it was not always possible to arrive at final conclusions. Thus, nucleic acid was incorporated in medium III on some evidence obtained with it in simpler media but, after the completion of the experiments described here, some additional experiments of rather short duration were made in which medium III was used with and without the nucleic acid. These experiments indicated that nucleic acid at the concentration used made the cells more granular than they were when it was omitted. Probably, therefore, with further experimentation, it will be possible to devise media that are simpler and still more satisfactory than those developed thus far.

[7] The authors are indebted to Dr. P. A. Levene of The Rockefeller Institute, who was kind enough to prepare and furnish this substance.

[8] Excellent results have been obtained with the whole thyroid gland in a medium containing only 1/5 of this quantity of hormone.

Preparation of Medium I.—To prepare 250 cc. of medium, calculate the amount of blood digest required to furnish 75 or 150 mg. nitrogen (*i.e.*, 12.5 or 25 cc. of a digest containing 600 mg. per cent nitrogen). Add to this 5 or 7.5 cc. homologous serum and 1.25 cc. of a 1 per cent solution of the sodium salt of phenol red. Then dilute with Tyrode's solution to 250 cc.

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[9] Obtained from the Connaught Laboratories, Toronto.

[10] 225 cc. of chloroform were used in making the digest for this work. A subsequent experiment has shown that by reducing this quantity to 50 cc., a digest of the same characteristics is obtained. Thus, when a given lot of blood was divided into two parts, one of which was treated with 225 cc. of chloroform, and the other with 50 cc., the following analytical results were obtained: Total nitrogen in the first digest, 439 mg. per cent; and in the second, 451 mg. per cent. Ratio of amino to total nitrogen, 0.445 in the first, and 0.477 in the second. As yet no comparative tests of these digests for their ability to support cell life have been made.

[11] In case a glass electrode is not available, it is advisable to add the pancreatin before adjusting the pH, and then to add N/1 NaOH solution until the mixture is just alkaline to phenolphthalein paper.

Preparation of Media II, III, and IV.—Stock solutions containing the most stable and inexpensive constituents at concentrations four times as great as those desired in the final medium are prepared first. These stock solutions are sterilized by passing them through a Berkefeld filter. They may be preserved at ice box temperature for 4 or 5 weeks. Solutions of the less stable and more expensive constituents are prepared in small quantity at frequent intervals. These are combined from time to time with small amounts of the stock solution to make the complete media. For organ cultivation, it is advisable to prepare a liter or more of the stock solution at a time. For culture work, much smaller quantities are advised. The freezing point of the medium should be determined before it is used and should lie between −0.62 and −0.66°C. The pH should be adjusted between 7.4 and 7.6. All media when completed should be sterilized by Berkefeld filtration.

To Prepare 1 Liter of Stock Solution for Medium II.—Calculate the volume of blood digest required to give 1200 or 2400 mg. nitrogen as desired (*i.e.*, 400 cc. of digest containing 600 mg. per cent nitrogen if a final medium containing 60 mg. per cent nitrogen is to be used). Measure out this volume and add:

Solution of sodium salt of phenol red, 1 per cent	20.0 cc.
Insulin, Squibb's (10 units per cc.)	0.4 cc.
Thyroxine-hemin solution containing 1 mg. thyroxine and 4 mg. hemin in 100 cc.	4.0 cc.
Potassium iodide, 2.8 per cent, diluted with water 1:100	18.6 cc.
Double strength Tyrode's solution	22.2 cc.
Cysteine hydrochloride, solid	360.0 mg.
Isotonic sodium bicarbonate solution, 1.4 per cent	6.8 cc.

Then dilute to 1 liter with Tyrode's solution modified to contain 300 mg. per cent glucose.

To Prepare 250 Cc. of Medium II.—Take 62.5 cc. of the stock solution just described and add:

	cc.
Ascorbic acid-glutathione solution (see below)	1.5
Vitamin B_1 (0.1 mg. per cent solution betaxin)	0.25
Vitamin B_2 (1.0 mg. per cent solution riboflavin)	0.85
Vitamin A serum, containing 1400 international units per cc. (or that amount calculated to furnish 250 international units)	0.18

Then dilute to 250 cc. with Tyrode's solution containing 300 mg. per cent glucose.

To Prepare 1 Liter of Stock Solution for Medium III.—Calculate the volume of blood digest needed to supply 2400 mg. nitrogen (or 1200 mg. nitrogen, if a final medium containing 30 mg. per cent nitrogen is desired). Add to this:

Tryptophane	200 or 400 mg.
Solution of sodium salt of phenol red, 1 per cent	20.0 cc.
Insulin, Squibb's (10 units per cc.)	0.4 cc.
Thyroxine-hemin solution containing 1 mg. thyroxine and 4 mg. hemin in 100 cc.	4.0 cc.
Cysteine hydrochloride, solid	360.0 mg.

Potassium iodide, 2.8 per cent solution diluted 1:100 with water	18.6 cc.
Urea	96.0 mg.
Vitamin B_1 (0.1 mg. per cent solution betaxin)	4.0 cc.
Vitamin B_2 (1.0 mg. per cent solution riboflavin)	13.6 cc.
Witte's peptone, 7.5 per cent solution in water	24.8 cc.
Sodium glycerophosphate	2.3 gm.
Glycerine, Kahlbaum's sp. gr. 1.23	8.0 cc.
Glucose	3.0 gm.
Sodium bicarbonate	270.0 mg.
Water, triple distilled	243.0 cc.

Then dilute to 1 liter with a Tyrode's solution from which the glucose has been omitted, and which contains sodium chloride at a concentration of 7.78 gm. per liter.

To Prepare 250 Cc. of Medium III.—Take 62.5 cc. of the stock solution just described and add:

Ascorbic acid-glutathione solution	1.5 cc.
Vitamin A serum containing 1400 international units per cc. (or that amount calculated to furnish 250 international units)	0.18 cc.
Antuitrin, Parke-Davis	0.5 cc.
Adrenalin chloride,[8] 1:1000 solution	0.25 cc.
Pitressin,[8] pituitary hormone, Parke-Davis	0.25 cc.
Eschatin,[8] suprarenal cortex hormone, Parke-Davis	0.25 cc.
Thymus nucleic acid dissolved in Ringer's solution with the aid of a few drops of N/1 NaOH	50.0 mg.
Water[12]	7.0 cc.

Then dilute to 250 cc. with Tyrode's solution containing 300 mg. per cent glucose.[10]

Phenol Red 1 Per Cent Solution of the Sodium Salt.—Weigh 1.000 gm. of phenol red. Grind this in a mortar with 28.2 cc. of exactly N/10 NaOH solution until it is all dissolved. Dilute to exactly 100 cc. with water, using a part of the water to transfer the dye to a graduated flask.

Thyroxine-Hemin Solution (Prepared as Described by Vogelaar and Erlichman).[8]—To 5 mg. thyroxine, add 6 cc. absolute alcohol, 2 cc. of 1 per cent NaOH, and 2 cc. water. Boil down to 3 cc. Add water to 10 cc. Then add 20 mg. hemin. Dilute 1:50 with water to obtain a solution having 1 mg. thyroxine and 4 mg. hemin in 100 cc.

Double Strength Tyrode's Solution.—A solution containing all the constituents of Tyrode's solution at twice the usual concentration. Sterilize by filtering.

Tyrode's Solution Modified to Contain 300 Mg. Per Cent Glucose.—Follow directions for making ordinary Tyrode's solution, reducing the sodium chloride to 7.78 gm. per liter and increasing the glucose to 3 gm. Sterilize by filtering. It is sometimes convenient to make this solution without adding the glucose since such a solution can be preserved for a few days in the ice box without filtering. Then the glucose may be added as each stock solution or medium is made.

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[12] The stock solution for medium III is hypertonic. Therefore water is added in making the medium. The stock solution cannot be made isotonic unless the volume of digest required does not exceed 554 cc.

Ascorbic Acid-Glutathione Solution.—Dissolve 40 mg. glutathione (Hoffman-La Roche) and 10 mg. crystalline vitamin C (natural, Abbott Laboratories) in 20 cc. Ringer's solution. Sterilize by passing through a 1 inch Berkefeld filter. The presence of glutathione is necessary to stabilize the vitamin C and protect it from oxidation. The solution should be made fresh every 10 days or 2 weeks and preserved in the ice box.

Witte's Peptone Solution, 7.5 Per Cent.—15 gm. of Witte's peptone is added to 200 cc. triple distilled water in a pressure bottle. Then this is autoclaved for 15 minutes at 30 pounds pressure or 15 pounds gauge pressure.

Vitamin A Serum.—To prepare vitamin A serum, a potent concentrate of vitamin A must be obtained. This may be prepared as described by Baker[13] or it may be obtained commercially.[14] As soon as this concentrate is received, it should be divided into small lots of approximately 0.75 cc. each, and sealed in small tubes under an atmosphere of CO_2.

Before the vitamin is incorporated in the medium, it must be dissolved in serum. To do this, place approximately 0.5 cc. of the concentrate in a small Erlenmeyer flask and add 30 cc. of serum. Shake the flask violently for 20 or 30 minutes in a shaking machine so as to obtain a finely divided suspension of the vitamin concentrate. Then allow the mixture to stand overnight at room temperature. The next day, filter the serum through a Seitz clarifying filter, and then sterilize it by passing it through a 1 inch Berkefeld filter. Sera containing as much as 1800 to 2600 international units of vitamin A per cc. may be obtained in this way. The amount dissolved varies with the individual sera and the species of animal from which it is taken.

To protect the vitamin A serum from oxidation it is divided into portions 1 to 2 cc. in volume, distributed in small glass tubes, and sealed under CO_2. If all the oxygen is removed, the vitamin serum will retain its original potency for 3 or 4 months. When the serum is kept in stoppered tubes without removing the oxygen, approximately half the vitamin is lost in a week. Moreover, the resulting oxidation product is somewhat toxic.

The concentration of vitamin A in the serum may be determined by a method worked out in this laboratory by La Rosa. The procedure depends on the development of the typical blue color of the Price-Carr reaction. This is changed to a purplish red color on heating. The latter color is compared with a series of standards made up of sodium alizarin sulfonate. Details of the method are described by Parker.[15]

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[13] Baker, L. E., *Proc. Soc. Exp. Biol. and Med.*, 1935, **33**, 124.

[14] That used throughout this work was prepared and donated for this purpose by the Abbott Laboratories.

General Procedure Used in Testing the Media

The procedure used in testing these media for their ability to maintain fibroblasts was as follows: Cultures from a 26 year old strain of chicken heart fibroblasts were embedded in Carrel D-3 flasks in coagula containing 0.25 cc. chicken plasma and 0.75 cc. of the medium being used. To remove the serum from this coagulum, the cultures were washed on the following day and every 2 days thereafter for 2 hours at 37°C. with 2 cc. of the medium. Then this wash fluid was withdrawn and 0.5 cc. of new medium was supplied. The serum originally present in the coagulum disappeared under this treatment within 12 to 14 days.[16] The washing was continued, nevertheless, throughout the entire period of cultivation. Before the flasks were sealed, the pH of the medium was brought to 7.4 by using a gas mixture containing 3 per cent CO_2, 21 per cent O_2, and 76 per cent N_2. To ascertain the effect of the various media, the cells were examined microscopically at frequent intervals. Then, at the end of the cultivation period, which extended from 43 to 56 days, the vitality of the cells and their ability to proliferate were tested by transplanting them into a growth-promoting medium (plasma and embryo juice). A sister colony was cultivated in each case in a control medium the nature of which is indicated under each experiment.

RESULTS

Medium I.—When tested in the manner just described, medium I was found to be an excellent maintenance medium. Fibroblasts cultivated in it remained alive and in good condition for 43 days.[17] During the first few days, *i.e.*, while considerable serum was still present in the coagulum, the cells proliferated at a very slow rate. After the concentration of the serum was reduced to that of the nutrient fluid, they were maintained with little or no proliferation. Then, when a little plasma was added on the 43rd day, to reinforce the coagula, the cells began to proliferate again. Control colonies that were cultivated in blood digest and Tyrode's solution died soon after all the serum had been removed from the coagulum. The colonies kept in digest supplemented with serum at 1 per cent concentration lived longer than those kept in digest alone, but not as long as those that received serum at 2 or 3 per cent concentration with the digest. Photographs illustrating the condition of the cells that were cultivated in blood digest alone, in blood digest supplemented with 1 per cent serum, and in blood digest supplemented with 2 and 3 per

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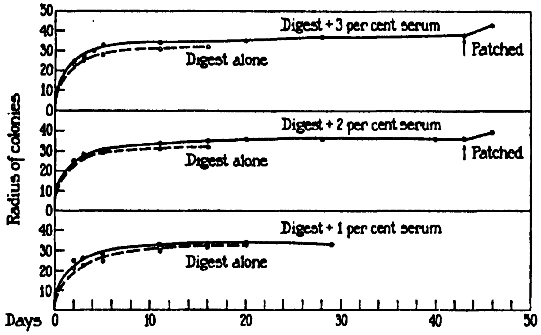
[15] Parker, R. C., The methods of tissue culture, New York, Paul B. Hoeber, Inc., 1938.

[16] To determine the length of time required to remove all this serum, a large number of coagula were prepared in Carrel flasks using several different samples of plasma diluted with Tyrode's solution to 25 per cent concentration. Then these were washed with Tyrode's solution in exactly the same manner as the cultures were washed with the medium. Each washing was followed by an incubation period during which 0.5 cc. of Tyrode's solution remained on the coagulum. After the third, fourth, fifth, sixth, and seventh washings some of the coagula were ground and the expressed fluid was analyzed for nitrogen. The organic matter was destroyed by digesting with sulfuric acid and hydrogen peroxide in the presence of selenium oxychloride, and the ammonia was determined by nesslerization. It was found that approximately two-thirds of the serum was removed by each washing and incubation period. After five washings, nitrogen equivalent to 0.001 cc. of serum was found. After seven washings, the largest amount of serum found was 0.0002 cc. Therefore, it is probably safe to assume that the serum is reduced to a negligible quantity by the end of 12 days' cultivation, and is completely removed soon after the end of the 2nd week.



TEXT-FIG. 1. Experiment 10816-C. Comparison of the rate of growth and the duration of life of fibroblasts cultivated in blood digest, supplemented with 1, 2, and 3 per cent serum, with that of sister colonies cultivated in blood digest without serum. Nitrogen concentration of the blood digest, 60 mg. per cent. The increase in growth on the 43rd day is due to patching the coagula with a small amount of plasma. Radius in mm. × 16.

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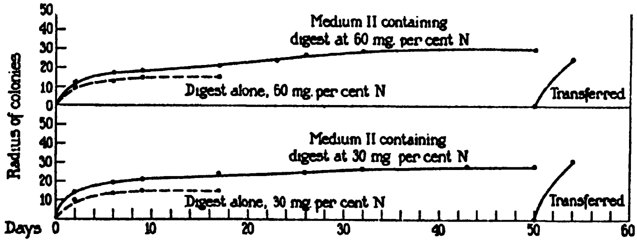
[17] In another experiment in which blood digest and Tyrode's solution were the only substances supplied in the nutrient fluid but in which the coagulum was reinforced once in 2 or 3 weeks by adding 2 drops of plasma, the tissue remained alive and in good condition for 70 days and proliferated again on being transferred to a growth-promoting medium.

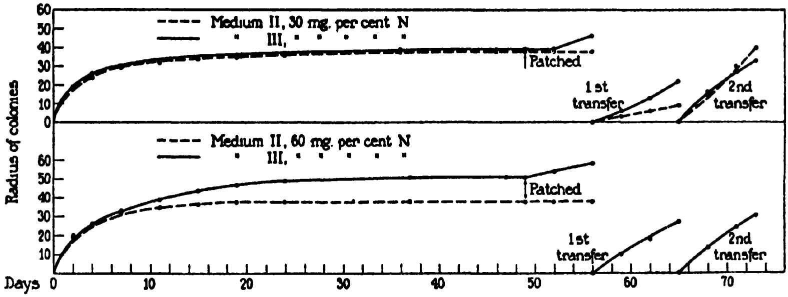
cent serum (medium I) are shown in Figs. 1 *a* to 1 *d*. Growth curves showing the duration of life of these cultures and that of the control colonies are shown in Text-fig. 1.

Medium II.—Chicken heart fibroblasts cultivated in medium II lived for 50 days. The control colonies, cultivated in blood digest diluted with Tyrode's solution to the same nitrogen concentration as that used in the medium, died during the 3rd week of cultivation. The cells in the experimental medium remained in good condition for 6 weeks. During the 7th week of cultivation, the cells at the periphery of the colony became somewhat scattered and began to look starved. As it seemed probable that longer cultivation in this medium would not be feasible, the colonies were transferred on the 50th day to a new coagulum and given growth-promoting nutrients. Active proliferation ensued. It would seem, therefore, that this medium can maintain the cells for a considerable time, but not indefinitely. Photographs illustrating the condition of the cells in medium II when made with digest at a nitrogen concentration of 30 mg. per cent, and also when containing digest at a nitrogen concentration of 60 mg. per cent, are shown in Figs. 2 *a* and 2 *b*. Growth curves showing the duration of life of colonies cultivated in these media and that of sister cultures kept in digest and Tyrode's solution are shown in Text-fig. 2. The second curve in each case is that of the experimental colony after it was transplanted and given a growth-promoting medium.

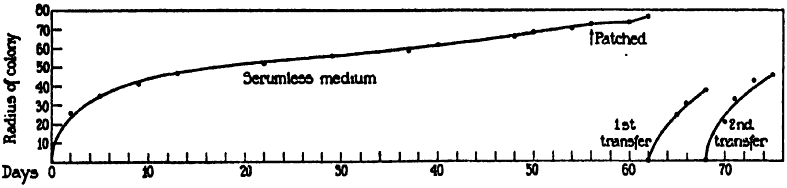
Medium III.—This was devised in an attempt to improve medium II. Four of the ten new constituents that were added, antuitrin, tryptophane, Witte's peptone, and sodium glycerophosphate, were found, when added separately to medium II, to improve the nutritive and maintenance value of that medium. Beneficial action was also observed with the other six substances when they were used together. But the differences observed when each was tested separately were too small to constitute definite proof that they were all essential. To illustrate the effect of the complete medium, an experiment is cited in which a comparison was made of the maintenance of power of medium III and that of medium II already improved by the addition of antuitrin and tryptophane. Sister colonies of fibroblasts were cultivated in these two media for 49 days. Almost from the begin-



Text-Fig. 2. Experiment 10835-C. Comparison of the rate of growth and duration of life of fibroblasts, cultivated in artificial medium II, with that of sister colonies cultivated in blood digest and Tyrode's solution, showing growth of the former after transplantation on the 50th day into a growth-promoting medium. Nitrogen concentration of the blood digest, 30 and 60 mg. per cent.



Text-Fig. 3. Experiment 10915-C. Comparison of the rate of growth and the duration of life of fibroblasts cultivated in artificial medium II to which antuitrin and tryptophane have been added, with that of sister colonies cultivated in artificial medium III showing growth in all cases in medium III and in some cases in medium II after transplantation on the 56th day into a growth-promoting medium. The increase in the rate of growth on the 49th day was due to patching the coagulum with a small amount of plasma. Nitrogen concentrations, 30 and 60 mg. per cent.



Text-Fig. 4. Experiment 11004-C. Curve showing the rate of growth and duration of life of a colony of fibroblasts cultivated for 56 days in an artificial and serumless medium (medium IV), showing growth after transplantation on the 62nd day to a growth-promoting medium. Nitrogen concentration, 60 mg. per cent.

ning of the experiment, the colonies in the more complete medium presented a better appearance. The tissue was thicker and the cells larger and clearer than were those cultivated in the simpler medium. In the experiments in which the digest was used at a nitrogen concentration of 60 mg. per cent, an exceedingly slow growth was observed. After 7 weeks of cultivation, the cells in the simpler medium began to look starved, as they had in the experiments with medium II cited above, while those in the more complete medium still seemed to be well nourished. On the 49th day, a small amount of plasma was added to reinforce the coagula. The colonies that had been given the more complete medium responded to this treatment by increasing in size. Those given the simpler medium did not respond. On the 56th day, the colonies were transferred to new coagula and given growth-promoting nutrients. All of those that had been cultivated in medium III grew actively, while only 25 per cent of those that had received the simpler medium were able to proliferate. Growth curves showing these results are reproduced in Text-fig. 3. Photographs illustrating the condition of the cells in medium III are shown in Fig. 3.

Medium IV.—This medium is serumless. It differs from medium III only by the omission of vitamin A and that small amount of serum that was required to dissolve it. Vitamin A had been incorporated in media II and III because it is a normal constituent of serum and because it had also been found to be an essential constituent of artificial, growth-promoting media. But no evidence indicating that it is essential to maintenance has been obtained. To ascertain, therefore, if it, and the serum that had been used to dissolve it, might be eliminated from the maintenance medium, an experiment was made in which sister colonies of fibroblasts were cultivated in medium III made up with and without vitamin A. The colonies that were kept in the serumless medium lived for 56 days. And the cells within those colonies remained a little cleared and appeared to be in better condition than those that had the vitamin and serum at their disposal. On the 56th day of cultivation, a little plasma was added to reinforce the coagula. Then on the 62nd day the cells were transplanted and given a growth-promoting medium. Those that had been maintained in the serumless medium as well as those that were given the medium containing the vitamin responded by proliferating actively. A curve showing the duration of life and the slow growth of one of the colonies in the serumless medium is reproduced in Text-fig. 4. The second and third curves show the growth of this colony on two successive transfers. A photograph illustrating the condition of the cells cultivated in the serumless medium is given in Fig. 4. As will be seen, the cells in this medium have become quite large and show less polarity than those in the other media. However, when they were transplanted and given growth-promoting nutrients, they reverted immediately to their original form.

SUMMARY

Several media designed for maintaining the life of cells and organs outside the body have been described. Cultures from a pure strain of fibroblasts have been maintained in these media in vital condition and with little or no proliferation for periods varying from 43 to 56 days.[18] One of these media is very simple, inexpensive, and easy to prepare; and one is serumless.

[18] 12 to 14 days should be deducted to calculate the time the tissues lived in the absence of serum.

EXPLANATION OF PLATE 19

FIG. 1 *a*. Control culture. Fibroblasts cultivated 32 days in blood digest and Tyrode's solution, showing degenerated cells. × 206.

1 *b*. Fibroblasts cultivated 32 days in blood digest, Tyrode's solution, and 1 per cent serum, showing cells in the process of degeneration. × 206.

1 *c*. Fibroblasts cultivated 38 days in blood digest, Tyrode's solution, and 2 per cent serum (medium I). × 206.

1 *d*. Fibroblasts cultivated 38 days in blood digest, Tyrode's solution, and 3 per cent serum (medium I). × 206.

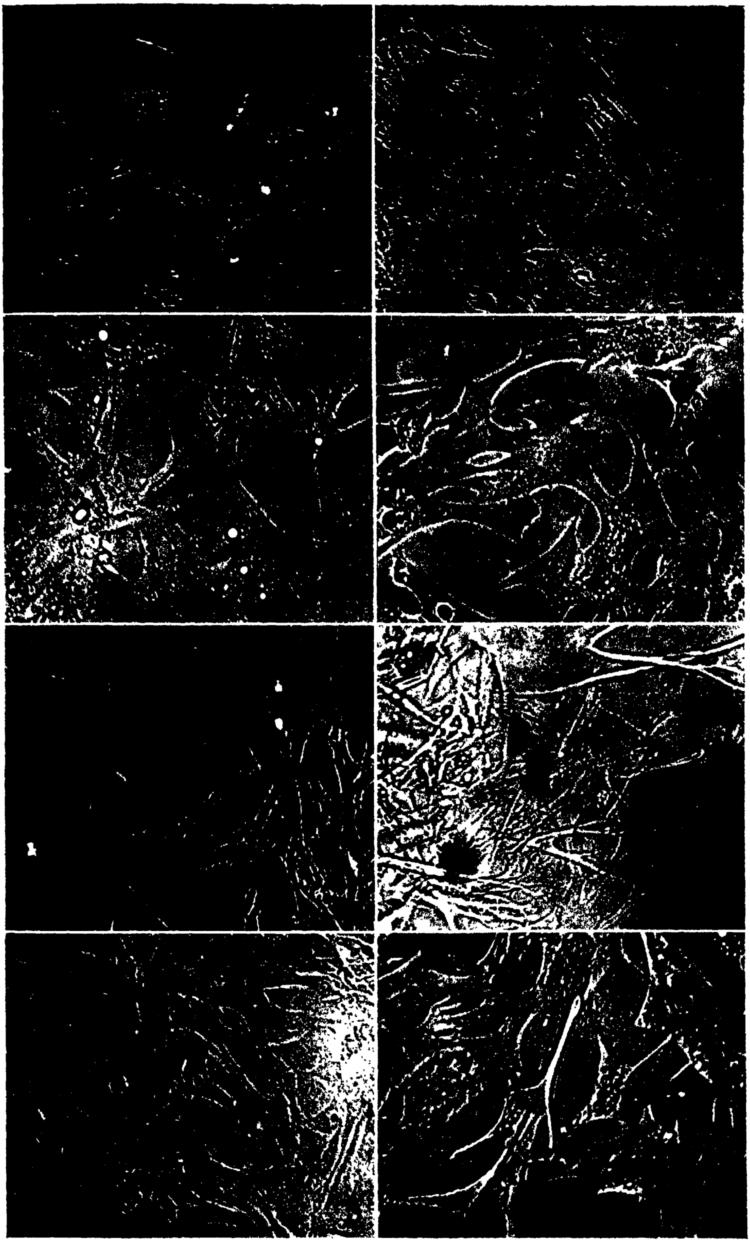
Nitrogen concentration of the blood digest, 60 mg. per cent in each case.

FIG. 2 *a*. Fibroblasts cultivated 37 days in medium II containing the blood digest at a nitrogen concentration of 30 mg. per cent. × 206.

2 *b*. Fibroblasts cultivated 32 days in medium II containing the digest at a nitrogen concentration of 60 mg. per cent. × 206.

FIG. 3. Fibroblasts cultivated 32 days in medium III containing blood digest at a nitrogen concentration of 60 mg. per cent. × 206.

FIG. 4. Fibroblasts cultivated 56 days in a serumless medium (medium IV), containing the blood digest at a nitrogen concentration of 60 mg. per cent. × 206.



(Baker and Ebeling: Artificial and serumless maintenance media)

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PHYSIOLOGICAL CONDITIONS EXISTING IN CONNECTIVE TISSUE

I. The Method of Interstitial Spread of Vital Dyes

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Plates 12 to 14

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The nutrition of tissues manifestly depends upon the interstitial dispersion of materials of varying constitution which, after escaping from the blood vessels and passing among the cells, return again to the blood, in part directly, in part indirectly through the lymph. That an extravascular movement of water and other substances exists, no one doubts, but how this transport takes place no one knows. Do actual spaces exist between the formed elements of the tissues? If so, is there a circulation of tissue fluid through them? The present paper contains the report of some experiments designed to throw light upon this problem.

In the course of earlier studies (1–3) upon the permeability of the lymphatics it has been observed that vital dyes intradermally injected at the tips of the ears of white mice enter directly into a superficial plexus of lymphatic capillaries, through channels torn by the injecting needle, and pass on the lymph to the base of the ear. The dye-containing channels stand out sharply in the colorless surrounding tissue. Invariably within a few minutes dye escapes all along them and passes into the tissue in situations far from the site of injection as well as near by. In proportion as this happens the dye-containing vessels lose their sharp outline; it becomes fuzzy, and at length obscured. Physiological changes taking place in the ear affect the rate and extent of this movement of dye into the tissues (1, 2).

It should be possible to learn much about the transport of substances of large molecule through the tissues by observing the movement of certain vital dyes under high magnification as they pass into unharmed, untouched regions of the ear at a distance from the injection site. Further, by the same means, it should be possible to gain some knowledge of the structure and nature of the interstitial spaces.

Methods

For the experiments to be described here we employed a vital dye pontamine sky blue[1] which has been used in our previous work on the permeability of lymphatics (1–7) and for its purpose has been injected intravenously and intralymphatically in mice and rabbits, and intradermally in human beings. It is an acid vital dye approximately 990 in molecular weight. It does not stain the formed elements of the tissues of the living animal during the periods required for the experiments. It is therefore specially suited for studies on the passage of substances through tissues.

The method for the purification of pontamine sky blue and the preparation of solutions has been given in a preceding paper (7). Aqueous solutions (21.6 per cent), isotonic with blood, were made up and subsequently diluted with Tyrode's solution or Locke's solution to the desired color content. For the present work, 2.0 and 10.8 per cent solutions were used. Both the solutions, though isotonic with blood, as determined by freezing point determinations, caused fluid to assemble in the tissues within a few minutes after their escape into them. This occurrence proved a great advantage in ways which will be evident below.

For injection purposes mice of 25 to 30 gm. body weight were anesthetized by the intraperitoneal injection of a 2 per cent solution of sodium luminal (0.125 cc. per 10 gm. of body weight), and placed upon plasticine moulds supporting the animal in such a manner that it lay prone with both ears horizontally spread on white porcelain placques (8). The ears were examined under a binocular microscope by light reflected from an arc lamp and passed through cooling filters as already described (9).

With a very sharp dissecting needle, ground as finely as possible, a minute skin puncture was made near the tip of the ear, under a lens that magnified about 15 times. Through the puncture a platinum-iridium, gauge 30, hypodermic needle was inserted and approximately 0.005 cc. of one of the dye solutions was introduced under slight pressure. There resulted a bleb of dye and even as it formed some of the colored matter entered the torn superficial lymphatic capillaries. The slight pressure was maintained by continued injection until the coloration had extended in one or more of the draining lymphatics to a point about one-third of the way to the base of the ear. All this was done very slowly, in the course of about 30 seconds, to obviate dilatation by pressure of the injected channels. There was no visible widening of the lymphatics, nor should any have occurred under the circumstances. When the needle was finally removed, the dye lay partly in the lymphatics and partly in a bleb at the tip of the ear, from which more passed into the lymphatics afterwards. During the succeeding 10 minutes the color moved slowly along the lymphatic capillaries toward the base of the ear

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[1] Du Pont Chemical Company.

As soon as the injection was completed and before any further extension of dye along the lymphatics had taken place, a layer of neutral paraffin oil was lightly painted on the ear to increase visibility, and immediately the plasticine mould holding the animal was transferred to the stage of a Leitz ultropak microscope for observation at magnifications ranging from 220 to 900 diameters. Illumination was obtained either from the 8 volt, 0.6 ampere lamp in the illuminator of the ultropak microscope, or by projecting the cooled light of a carbon arc lamp through the illuminating attachment for light from outside sources. The carbon arc was used for taking photographs which was done with a Leica camera supported upon a Leitz camera attachment.

The Characteristics of Dye Escape from Lymphatics into Normal Tissue.—The escape of vital dyes from superficial lymphatics of human skin and the mouse ear, as witnessed with the unaided eye and at low power magnification, has already been described (1–6) and photographed. Under these conditions the sharp outlines of the dye-containing channels become blurred and fuzzy as the color appears outside the vessels. The bands of extravascular coloration gradually become broader until they touch each other and the tissue becomes diffusely stained.

When observed at a magnification of 900 diameters the vital dyes first appear as diagrammatically sketched in Fig. 1*a*, as sharply defined, almost linear extensions of color, at times wavy and parallel with the course of the channel, at other times like bristles radiating outwards like the ribs of a fan. These hair-like projections of color maintain their contours for several minutes, until gradually their margins blend in a generalized, diffuse staining.

As dye escape continues, the bristles and wavy lines become darker in color and others, of similar shape but pale and indistinct, make their appearance farther from the vessel, as shown diagrammatically in Fig. 1*b*. In more than 200 experiments in which the phenomena of dye escape have been observed we have been unable to perceive actual outward movement of the color. The eye first becomes aware of a few very pale, wavy lines which gradually become darker, while at the same time neighboring lines further from the channel suddenly appear, faintly and lightly colored. These in turn become more easily visible, while others appear still farther away and fainter. But there is no visible creeping outward of the color. The transition to visibility can best be compared to a "fade-in" in moving pictures or to the development of a photographic print.

In scores of instances we have watched the escape of dye from the lymphatic capillaries while micro probes, held in the Chambers device, were used to make pressure upon, or to manipulate, the colored projections as they appeared. For 8 to 14 minutes and sometimes longer, when 2 per cent pontamine sky blue was used, or for shorter periods when stronger solutions were employed, local pressure did no more to the colored lines of bristles than to bend and twist them back and forth without changing their outlines or diminishing their intensity. When two micro probes were placed together upon a colored projection and then separated under pressure, the color did not move away in the direction of the movement of the probes, as would the contents of a blood vessel, lymphatic or any other open channel. It did not behave as though free in a fluid which could be pressed away by the micro probes. Evidently the dye had extended outward from the lymphatics and was situated on or between the surfaces of preformed structures which, as our subsequent work has shown, were probably connective tissue fibrils.

After the passage of a little time the appearance of the hair-like projections changed in all of the experiments, over 300 in number. The projections became broader, not quite as deeply stained, and their contours less sharp. This was true regardless of the concentration of the dye solutions used, the changes occurring more rapidly the more concentrated the dye; for example, in 3 to 6 minutes when 10.8 per cent pontamine sky blue in Locke's solution was employed, and in 5 to 12 minutes when 2 per cent solution was used. During this phase of alteration the color could not be squeezed from the paling, bristly projections as they were bent or prodded about by a micro probe, though they were much less sharply outlined as indicated in the diagram of Fig. 1*c*. Soon the intervening space between the colored projections was noted to have become diffusely blue. There followed a variable period in which gentle pressure with the micro spatula failed to lighten the color of these diffusely stained areas or to alter the hair-like projections of color which by this time had become faint in hue. This condition of affairs will be referred to as the "second phase" of dye escape and is depicted in Fig. 1*d*. When 10.8 per cent pontamine sky blue solution was used, the second phase appeared on the average during the 5th minute after dye escape was first perceptible, and it persisted for several minutes. Then, quite suddenly, one found somewhere in the ear that gentle pressure over a pale blue, hair-like projection, or over the diffusely blue regions between, squeezed the color from the point at which pressure was exerted. And where this had happened the colored, hair-like projections disappeared within the next few minutes, as indicated in Fig. 1*e*. It was plain that now free fluid stained with the dye was present in the tissues. Once this had happened the further spread of the dye was no longer accomplished by a linear, bristly staining but by a diffuse coloration obviously due to dye-containing fluid. Even at its advancing edge the color could be freely moved about with a micro probe.

To summarize, there always appeared first a broadening of the hair-like projections, as though some of the dye had spread in the interfaces between the connective tissue fibers. Then appeared the second phase in which diffuse blue was seen between the colored projections, which still remained visible on pressure. In the last stage light pressure applied to the skin with a micro probe easily moved the blue interstitial fluid through the tissues.

When lymphatics filled with dye were forcibly ruptured by simultaneously squeezing from opposite directions, with two micro probes, the hair-like projections did not appear. Instead, the resulting ecchymoses of dye lay outside the channel like smooth-surfaced bulbs or polyps extending from the lumen of the vessel by thin stalks of dye, as diagrammatically shown in Fig. 1*f*. Obviously the dye-stained fluid had pushed the tissue apart and lay in a smooth-walled cavity.

The phenomena here described did not take place consecutively in all parts of the ear at once. In different regions of the ear all could be found at the same time. In order to compare the findings in different instances, we have confined our observations to the region of the middle third of the ear and have noted the earliest appearance of each phase of the dye movement. In each test we took the times of earliest dye escape from any lymphatic in this region with a stop watch, and that of the first appearance of colored, hair-like projections. The projections in various regions of the ear were then prodded with the micro probe. The time of appearance of the second phase as described above was next taken, as also that of the first appearance of freely movable color under the probe, indicating the presence of free fluid within the tissues in sufficient amounts to be recognized.

The times at which these events occurred when any given concentration of dye was used were remarkably constant, considering the qualitative and the subjective nature of such observations. Needless to say great individual variations occasionally appeared, but the method proved amply adequate to disclose the deviations which accompanied induced physiological or pathological changes in the ears. For example, in 20 experiments using 10.8 per cent pontamine sky blue solution, hair-like projections first appeared, on the average, in 1 min. 35 seconds. In 80 per cent of these tests the individual variations amounted to not more than 35 seconds. The earliest appearance of dye in the other 20 per cent occurred 1 minute and 5 seconds after injecting the lymphatics, the longest delayed after 2 minutes 40 seconds. Free fluid could first be demonstrated in about 9¼ minutes on the average, the variation from this average being only 2 minutes or less in 80 per cent of the cases. In the remaining 20 per cent of the tests free fluid was demonstrated at the earliest after 7 minutes and at the latest after 14 minutes.

It will be seen from the data to be given below that the time intervals at which these phenomena occurred varied greatly when the animals were subjected to the various experimental procedures. For example, in the ears of mice which had just been bled, free fluid could not be demonstrated on the average for 21 minutes after injecting the dye, the phenomenon appearing as early as the 16th and as late as the 40th minute. In animals recently killed free fluid could not be demonstrated at all. In Table I there is presented, in anticipation, a summary of these differences to serve as an aid in comparing the different photographs to be discussed below. The figures in the table show the averages of the time intervals after injecting the ear, at which the various phenomena were observed. Where the averages differed greatly from the extremes of the intervals, the latter are given in parentheses. The figures are not to be construed as quantitative, but they do indicate, in a rough way, the fact that the phenomena of the interstitial movement of dye, occurring in the normal ear, as already described, are delayed or hastened by changes in the physiological state of the animal.

The solutions made with batches of the purified dye prepared in different years showed slight differences in the speed of escape. All the experiments herein described were done with solutions freshly made up from a single batch of each dye, and only those experiments were compared in which a uniform concentration of dye was used.

The phenomena just described were witnessed under a magnification of 900 diameters. Figs. 2 to 7 are photographs of some of the happenings as they appeared in the ears of normal mice, magnified only 285 times. Under such conditions many of the changes could not be clearly seen and at best they are merely indicated in the photographs. To obtain these pictures, a 10.8 per cent solution of pontamine sky blue was employed, with result that the events occurred more rapidly than in the experiments in which a 2 per cent dye solution was used. Photographs *a* to *i* inclusive of Fig. 2 were taken at 2, 3, 4, 5, 6, 7, 14, 20, and 25 minutes respectively after the intro-

TABLE I

The Effect of Tissue Hydration, Dehydration, and Death upon the Interstitial Spread of Dye in the Mouse Ear

(A 10.8 Per Cent Solution of Pontamine Sky Blue)

	Normal mice	After intravenous injection of Tyrode's solution	After hemorrhage	After death
	min.	*min.*	*min.*	*min.*
First appearance of colored bristles	1½ (1–2¾)	1 (40 sec.–2 min.)	2½–3 (2–4)	3½ (3–4½)
First definite widening of colored bristles	3-6	These stages could not be seen as the coloring was sometimes diffuse from the beginning	4–6	7–8
Second phase (first appearance of diffuse color between bristles; color cannot be dislodged by pressure)	5-6 (4–7)		9 (7–15)	10 (7–16)
Free fluid (first demonstration of freely movable color)	9½ (7–14)	Occasionally from the beginning	21 (16–40)	None

The figures appearing in the table represent the average time, following injection of the lymphatics, at which the phenomenon in question appeared. In the instances in which the averages and extremes differed greatly, the latter are given in parentheses.

duction of the dye. In Fig. 3 the photographs were taken in more rapid succession at 1 minute intervals from the 2nd to the 10th minute. As result the process of dye escape is not as advanced as in the last three pictures in Fig. 2. The first three photographs of the latter show the early appearance of the colored bristles. They had become broader by the 5th minute when photograph *d* was taken, and in photographs *e* and *f* the second phase has been entered. The last three photographs of Fig. 2 show the diffuse blue color which, as demonstrated in other parts of the ear, could be pushed about through the tissue by the micro probe. In these pictures the round white discs are groups of hair follicle cells which stand out against the generally blue background. The purpose of the arrows in Figs. 2 and 3 will be indicated below. When Figs. 3*d* and *e* were taken the blue projections had become broad, 5 and 6 minutes after injecting the dye. The second phase was present when Figs. 3*f* and *g* were taken. Unfortunately the low magnification of the photographs fails to bring out the changes which had taken place in the appearance of the colored projections. It is to be stressed here that the individual hair-like colored projections can scarcely be identified in these low power photographs in which they constitute collectively the finest fuzzing of the gray borders seen about the lymphatics. They are extremely numerous and lie close together. Only the diagrams in Fig. 1 give a true idea of their appearance at higher magnifications and even in these sketches, for simplicity, we have not shown them to be as numerous as they really are.

The Nature of the Colored Projections

Why does the dye escaping from the lymphatics first appear as bristles or wavy lines extending from the channels and later color the surrounding tissues evenly?

The anatomical studies of others have demonstrated an intimate association of connective tissue fibers with the walls of lymphatic and blood capillaries. As early as 1894 Starling suggested that connective tissue fibers may serve to hold the lymphatics open in an edematous tissue (10, 11). More recently, Pullinger and Florey (12) have demonstrated that connective tissue fibers, if not actually part of the lymphatic capillary wall, are so associated with it physiologically that they act as a unit. Illustrations presented as Figs. 8 and 10 in the paper by these authors show connective tissue fibers branching out from the lymphatic capillary wall and running all about the vessels. Dye escaping from these channels and moving upon or between these fibers would appear like the colored projections we have described. Clark and Clark, in a study of the relation of lymphatic capillaries to blood vessels (13) have presented excellent photographs (Figs. 6 to 10 and Figs. 13 A and B in the paper referred to) which demonstrate the relationship of the connective tissue to the lymphatics.

In the last year Zweifach (14), studying the relationship of connective tissue fibers to blood capillaries by means of the Chambers micro dissection apparatus, has pictured their most intimate association with the capillary endothelium. In preparations vitally stained with Janus green, he found that certain fibers of the inner pericapillary reticulum, joined the endothelium of the capillaries. Surrounding this network, other, coarser fibers could be made out, the ends of which were partially embedded in the endothelial wall. Cells like fibroblasts, among which fine fibrils ran, surrounded the vessels completely and connective tissue fibrils extended directly to the endothelial surface. These structures, invisible in unstained tissue, were readily seen when Janus green was used. Zweifach's findings make it difficult to see how either fluid or particulate matter can pass out of a blood capillary without coming into intimate contact with the connective tissue fibers.

Jones (15), in discussing the innervation of the blood capillary, has stressed the relationship of the connective tissues to its wall. Bouin (16) has gone so far as to state that the embryonal capillary may be entirely reticular and that the cellular elements of the capillary may be so incomplete, even in adult tissues, that the current of blood is guided wholly by reticulum.

From the work of others just cited it is apparent that connective tissue fibers are similar in form and in relationship to the lymphatic capillary wall, to the colored projections which appear in the ear of the living mouse, when dye escapes from the lymphatics. Dye appearing upon or between these fibers, as just stated, would appear like the colored projections.

Artifacts and Optical Illusions.—To prevent misunderstanding it is necessary to describe and picture certain phenomena appearing during the escape of dye from the lymphatics which might be mistaken for the bristly and wavy lines just discussed.

In viewing at high power a lymphatic channel just filled with dye, a common optical illusion may appear either before or during the escape of dye from the channel. The refraction of light by the keratinized surface epithelial cells gives rise to the appearance of colored wavy lines apparently extending outward from the channel or along it. But these also extend across the lymph channels. Careful examination shows them following a definite pattern which can be found at any time after the intradermal injection of a colored substance. These lines do not appear when the walls of the lymphatics are in focus. They are larger than the colored bristles and wavy lines under discussion and lack their transient character. Fig. 4*a* shows the appearance of these artifacts, 2 minutes after injecting the lymphatics with dye and before its escape had begun. Fig. 4*b* shows the same lymphatic 3½ minutes later when dye escape was well under way. The heavy wavy lines appearing in these photographs are optical illusions which can be corrected by proper focussing.

In most of our experiments dye was seen escaping from lymphatics and extending along the walls of small or large blood vessels or nerves. Such an alignment of dye along blood vessels and nerves is evident in the photograph in Fig. 5, taken 7 minutes after injecting the dye. The arrows *a* in the figure show the dye on both sides of the lymphatic spreading along the outer walls of an arteriole which crosses the lymphatic; the arrows at *v* show dye running along the walls of a venule which also crosses the same lymphatic. The resulting spikes of color cannot be mistaken for the colored projections shown in Figs. 2 and 3. In Figs. 2*c*, 2*d*, and 2*g*, arrows, already referred to, have been placed to show the appearance of dye which has spread along the sheath of a nerve which crosses the lymphatic at right angles. The same phenomenon is indicated by the arrows in Figs. 3*d* and 3*e*. In Figs. 6 *a*, *b*, and *c*, slightly different aspects of the same phenomenon are shown. In photograph *a*, dye escape has just begun in the usual manner 2½ minutes after the injection. In *b* and *c*, taken 4 and 6 minutes after the injection, there are none of the bristly or wavy lines to be seen, the focus being imperfect, but the extensions of color along the vessels crossing the lymphatics are obvious. They should not be confused with the hair-like projections. These are much smaller and finer and are probably due to the presence of dye in the interfaces between the surfaces of contiguous connective tissue fibers as suggested above.

At times finger-like extensions of color appear to extend from the lymphatics, larger than the hair-like projections under discussion. These are visible under a low power binocular microscope at magnifications of 80 to 100, and are even distinguishable at magnifications of 15 or 20. They are about the size of the bundles of collagen fibers or of the intercellular matrix of fat cells of the hair follicles. Like the finer bristles and hair-like projections of color they show for a period sharply defined color which becomes lost later in the generalized diffuse staining of the ear. Fig. 7 illustrates some as they develop, together with the bristly and wavy lines. The photographs were taken at one minute intervals, beginning at the 4th minute after injection. The first three photographs (*a*, *b*, *c*) show the bristles of color as dye escape from the lymphatics began. They appear just as in the first two or three photographs of Figs. 2 and 3. Photograph *d* is slightly out of focus, and the two remaining (*e* and *f*) badly out of focus, all three showing optical illusions and incidentally some of the larger finger-like projections probably due to the spread of dye between large collagen bundles or along them (indicated by the arrows in Fig. 7*e*).

The Effect of Hydremic Plethora.—In the experiments described so far in this paper the vital dye pontamine sky blue escaped from the lymphatics into tissue previously normal. It seemed a matter of interest to determine next whether or not the addition of large amounts of fluid to the blood would lead to demonstrable changes in tissue state. It is well known that balanced salt solutions escape rapidly from the blood stream, in part into the subcutaneous tissues, after intravenous injection. What effect would the resulting condition have upon the interstitial movement of dye?

Thirty mice of about 25 gm., under luminal anesthesia, were injected into the tail vein with 0.6 cc. to 1.4 cc. of Tyrode's or Locke's solution in 2 to 10 minutes. About 1 minute after beginning the intravenous injections, 2 per cent or 10.8 per cent pontamine sky blue solution was injected into the lymphatics of the ear in the usual manner.

In contrast to the findings in normal ears, already described, a blue band of color appeared outside the lymphatics which showed fewer than normal of the bristly, wavy lines of color. In some regions the color could be moved about or squeezed away with the micro probe almost as soon as it appeared, while in others it could not, though the wavy lines were less prominent than in normal ear tissue. The photographs of Fig. 8 were taken during a typical experiment at 1½, 3, 4, 5, 6, and 7 minutes after injection. They can be compared with the first six photographs of Figs. 2 and 3, for the time intervals after injection at which the respective photographs were taken are roughly similar. It will be seen from the photographs of Fig. 8 that the dye escape was much more general. Evidently the manner of extension of the dye through the tissues had been greatly altered.

The Characteristics of Dye Escape from Lymphatics in Edematous Tissue.—Ears rendered edematous by inflammatory agents yielded similar, but more pronounced, findings.

On 30 occasions one or both ears of luminalized mice were painted with xylol. The usual intense reactive hyperemia (3) occurred in 1 or 2 minutes, followed in 10 to 15 minutes by edema. The ears assumed a thick, ground glass appearance within 15 minutes after applying the irritant. When pressed with a blunt instrument they "pitted on pressure" and if pricked with a sharp needle exuded droplets of clear fluid. At various intervals, from half an hour to 18 hours after painting both ears with xylol, the lymphatics of each ear were injected in the usual manner with the pontamine sky blue solutions. The first appearance of dye outside the lymphatics took the form of a diffusely colored band easily movable by pressure with the micro probes. The bristly or wavy lines of dye were absent in ears painted 30 minutes or an hour beforehand, in which edema was present and increasing; as also in ears painted 18 hours previously in which edema was probably decreasing.

Good photographs of the dye escape in the xylol-painted ears could not be taken at high magnification because of the edema. In these experiments the progress of dye from the lymphatics outward into the tissue seemed to occur solely by diffusion into the fluid.

The Progress of Dye through Relatively Dehydrated Tissues.—The experiments so far reported have shown that the movement of dye through intradermal tissues containing free fluid, in demonstrable amounts, is different from that in presumably normal tissues. In the normal ear the presence of free fluid has not been demonstrated and the escape of dye seems to take place along fibers, although, as mentioned earlier, we have not been able to see its actual extension along them.

The effect of a decrease in the water content of normal tissues upon the transport of dyes was now investigated.

The Characteristics of Dye Transport in the Tissues of Bled Mice

Mice of 25 to 27 gm. body weight, anesthetized with luminal, were bled 0.7 to 1.0 cc. by a technique described in a preceding paper (7). In 35 experiments the ears were injected with dye in the usual manner when the bleeding was almost completed, and its escape from the lymphatics was watched at high power and photographed as usual at a magnification of 285. The findings were consistent and they need not be dwelt upon at length.

In the bled mice the bristling, fibrillar appearance of the escaping dye was far more pronounced than in the normal ears, but it came to attention much more slowly and the colored bristles or wavy lines could be bent and twisted with the micro probes for a longer period without losing their contours. It was plain that the dye remained either between or spread upon the connective tissue fibers for a longer time. Occasionally the lines of color could actually be seen elongating as if a "creeping" of dye along or between fibers took place. In the experiments in which 10.8 per cent pontamine sky blue solution was employed the second phase of dye escape endured for a longer period than in normal ears (Table I). The colored projections lost their sharp outlines after approximately the same time intervals as in normal tissue, and diffuse staining appeared between them which did not yield to pressure with the micro probe; but the demonstration of free fluid by movement of color could not be obtained until 21 minutes had elapsed on the average (the range of individual variation being 16 to 40 minutes, as compared with 7 to 14 minutes in the normal). Free fluid eventually formed in the intradermal tissues in recognizable amounts, despite the dehydration incident to the hemorrhage.

The Movement of Dye through the Ear Tissue of Dead Animals.—The experiments just described showed that the removal of blood with the resulting dehydration did not essentially alter the mechanism of transport of substances through the tissue, but merely slowed the course of events. The hair-like projections of color which developed outside the lymphatics were more pronounced than ordinarily, and formed more slowly. It was a matter of interest to study next the movement of dyes through tissues in which no fluid exchange of ordinary sort was taking place, that is to say in the ears of mice recently killed with ether or chloroform.

The usual injections of 2 and 10.8 per cent pontamine sky blue solutions were made into the ears of 30 mice from 5 minutes to 5 hours after killing the animals with ether or chloroform. When the injection was done 5 minutes after the circulation in the ear had ceased, as observed under the binocular microscope, the escape of dye from the lymphatics was very slow. Bristles of color formed which were even more pronounced than in the ears of the bled animals: they stood out more clearly, were more deeply colored and shorter than in normal ears. They also remained visible for a longer time than in the tissue of living animals. The dye did not travel as far through the tissues as in the bled animals and not nearly as far as in the normal ones. This is true, as well, in the ears of animals dead ½ to 5 hours.

In Fig. 9 are six photographs typical of the changes. They were taken 4, 7, 10, 15, 20, and 25 minutes after injecting the lymphatics of an animal dead for 1 hour. The short, stubby, and heavily colored projections of dye, some of which are indicated by arrows in the figure, were exceedingly striking. A comparison with Figs. 7 and 2 will show how slow the dye escape was. Even after 20 minutes the colored lines, though now broader than when first seen, could be manipulated with the micro probe, bent and twisted back and forth without losing their contours, and the diffusely stained material lying between them could not be displaced by the pressure.

When 10.8 per cent pontamine sky blue was used, as in the experiments in which the photographs were taken, the second phase of dye spread appeared in these animals at about the 10th minute following the injection (the time varying from 7 to 16 minutes). In this respect they showed but little difference from the bled animals, dye diffusing away from the bristly wavy lines of color with only moderate delay. But this stage, in which diffuse coloration of the tissues took place but the color could not be moved about with the micro probe, endured long. Definite evidence of stained free fluid movable in the tissues was not obtained even after 45 minutes to

1½ hours, though heavy, diffuse staining had taken place. On pressure with the micro probe one indented the ear, with result that the color there became slightly paler, but the gentle pressure effected no displacement of color. Findings similar to these were obtained when layers of agar or gelatin 5 mm. in thickness and stained with dye were pressed upon in the same way under the microscope. The finding in the dead animal was characteristic of the second phase of dye escape, but the staining was more intense than usual.

DISCUSSION

The colored projections of dye extending from a lymphatic shortly after it has been injected are not optical illusions. When similar lymphatics are filled with India ink, hydrocollag, or Prussian blue, no such projections are seen. Instead the channels appear for hours just as they do when first injected. Their outlines remain sharp, and show the characteristic anatomical irregularities, indentations, and bosses, but never the hair-like extensions we have discussed. The observations reported here have been made in the ear of the mouse because of the excellent visibility of the tissues. We have observed similar phenomena in the skin of the leg and abdomen of mice, in the connective tissues of the testicle, scrotum, and conjunctiva of mice, and in the ears of rabbits. The findings seem applicable to connective tissue in general in so far as they have been studied.

The work of others, referred to earlier in the paper, has shown an intimate association of connective tissue fibers with the lymphatic capillary endothelium. The appearance and distribution of the colored bristles which come into view along the lymphatic vessels are precisely that of the connective tissue fibers which radiate out from these latter (14, 12), and it seems certain that the movement of dye into the tissue is conditioned by the form and structure of the fibers. The intimate association of connective tissue fibers with lymphatics and blood capillaries has long been known, but the fact that fibers are utilized as pathways along which substances may move through the tissues has not been recognized.

Although we have been unable to demonstrate the presence of free fluid in the normal tissues of the mouse ear, the possibility remains that it is present there in amounts which defy detection. The diffusion of pontamine sky blue might have been so slow that it failed to reach the free fluid before its own irritant action had called forth an edema. Suffice it to say here that experiments to be detailed in the accompanying paper show that this is not the case.

What can one infer from this work about the movement of substances other than dyes or of water? The findings of other experiments which bear upon this theme are reported in the accompanying paper. Discussion of the present findings will be deferred to that paper and further considered in relation to findings reported by us in earlier papers.

SUMMARY

The escape of a vital dye from the lymphatics of the ears of living mice and its subsequent movement through normal and pathological connective tissue have been observed at high magnification. The dye first appears outside such channels as bristly, wavy lines of color, which can be bent and twisted by pressure with a micro probe and spring back to their original positions when the pressure is removed, as if the dye were fixed upon or between some tissue elements. Our findings indicate that this is the case, that the bristly lines of color are formed by dye moving between connective tissue fibers or along them. With the onset of mild edema, such as the dye induces secondarily, the bristles disappear, the coloration becoming diffuse and freely movable with the micro probe.

When edema is induced before dye is introduced into the lymphatics, the character of its escape is wholly different. It first appears as a colored cloud, freely movable in the edema fluid, the manner of its passage into the tissues being completely changed.

In the ears of mice partly dehydrated by bleeding, or in those of dead animals, the bristly or wavy lines were more evident than in normal individuals. It was plain that dehydration did not change the mode of transportation of the dye through the tissue but merely emphasized some of the characteristics of its passage. In animals injected intravenously with large amounts of physiological saline, with result in the presence of more tissue fluid than usual, the colored bristles were seldom seen.

It is plain that connective tissue fibers serve indirectly as pathways for the transport of substances of large molecule.

We have not been able by the dye method to demonstrate the presence of any free fluid in the normal tissues of the mouse ear.

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EXPLANATION OF PLATE 12

FIG. 1. Diagrammatic sketch of the extravascular interstitial movement of a 2 per cent solution of pontamine sky blue following its escape from the lymphatics, as described in the text.

(*a*) Dye first appears as colored bristles (2 to 7 minutes).

(*b*) Color becomes more intense and bristles longer (3 to 10 minutes).

(*c*) Colored lines become broader (5 to 12 minutes).

(*d*) Second phase. Diffuse blue staining between bristles which cannot be dislodged by pressure. Bristles disappearing.

During this period color apparently fixed on tissue elements cannot be dislodged by pressure

(*e*) Diffuse blue cloud easily displaced with pressure (free fluid).

(*f*) Dye escaping from ruptured lymphatics, no bristles.

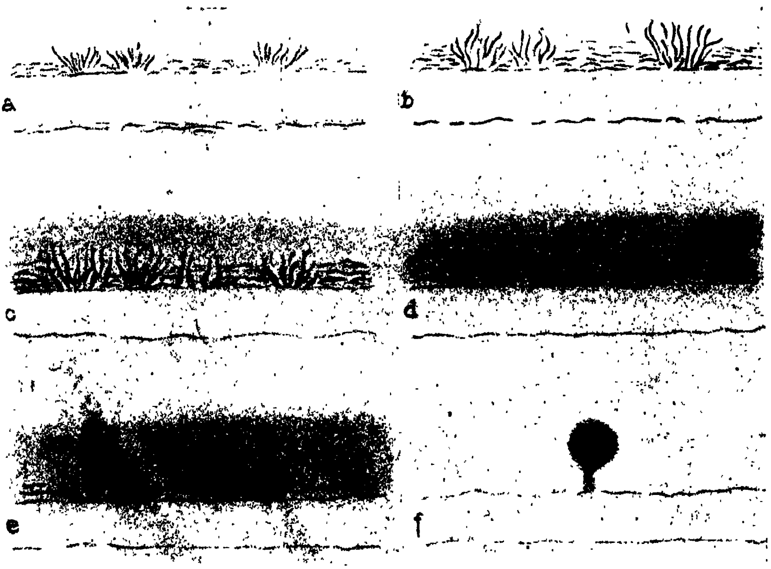

FIG. 1

(McMaster and Parsons: Physiological conditions in connective tissue. I)

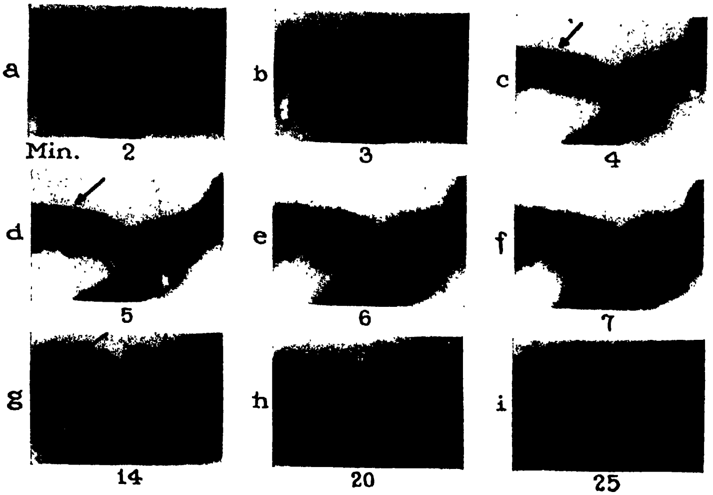

FIG. 2

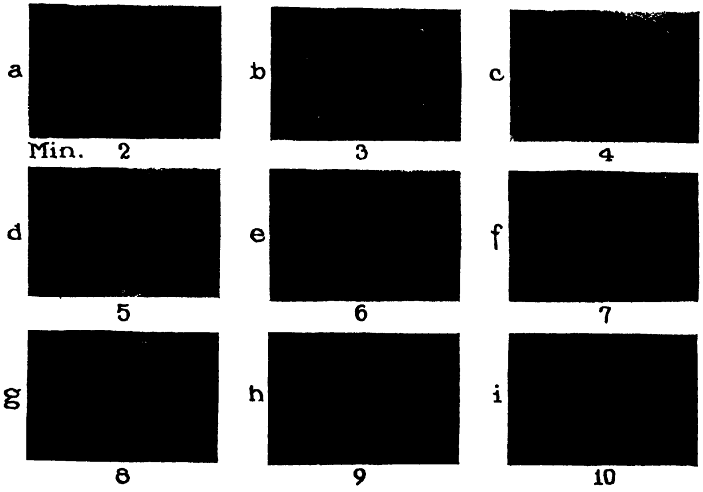

FIG. 3

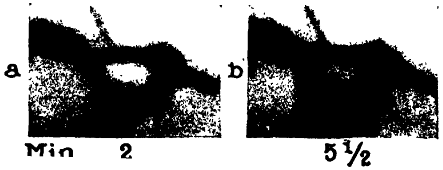

FIG. 4

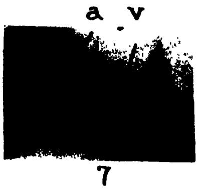

FIG. 5

(McMaster and Parsons: Physiological conditions in connective tissue. I)

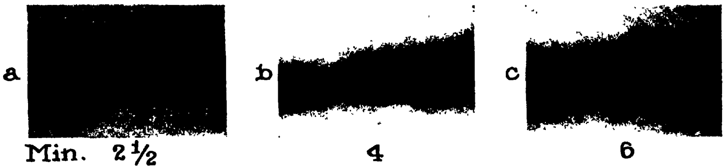

FIG. 6

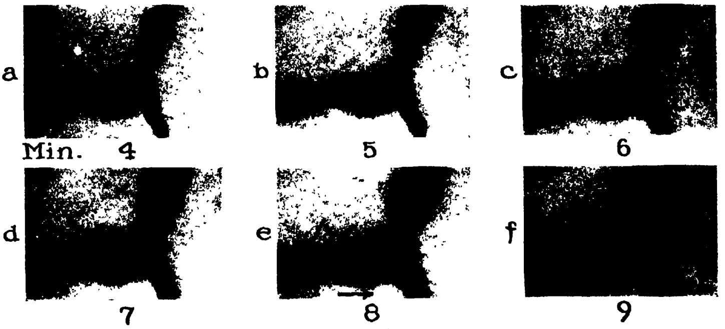

FIG. 7

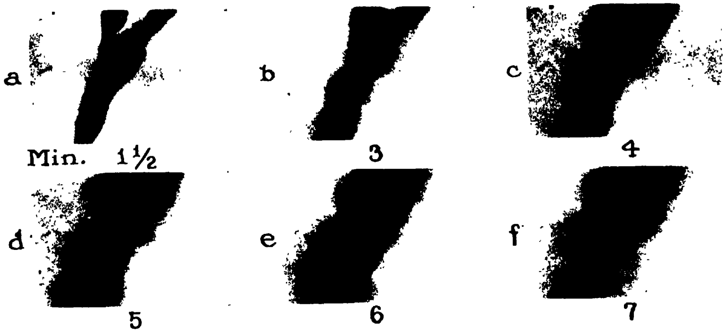

FIG. 8

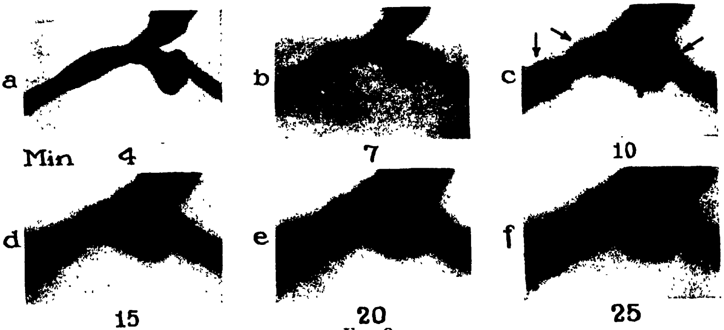

FIG. 9

(McMaster and Parsons: Physiological conditions in connective tissue. I)

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PHYSIOLOGICAL CONDITIONS EXISTING IN CONNECTIVE TISSUE

II. The State of the Fluid in the Intradermal Tissue

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The findings of the preceding paper indicate that substances of large molecule extend through connective tissue along connective tissue fibers (1). A vital dye, escaping from the lymphatics of the ears of living mice, could first be seen outside the channels as bristly lines of color extending from them apparently along the fibers. These lines of color can be bent and twisted by pressure with a micro probe and spring back to their original positions when the pressure is removed, as if the dye were fixed upon or between the fibers. Its subsequent movement seemed to be determined by their form and structure. The removal of body fluid from the animals by bleedings did not change the way in which the dye extended. By contrast it was completely changed by the presence of free fluid or edema in the tissues. Dye escaping from lymphatics into edematous tissue took the form of a diffusely colored band easily movable in the edema fluid when pressed upon by a micro probe.

Because of these facts, and because our observations failed to disclose the presence of free interstitial fluid in normal connective tissue it seemed important to study the movements of other dyes through normal tissues and those containing free fluid. Will dyes, differing in diffusibility and in chemical constitution from the one already employed, show the same phenomena in passing through the tissues; that is to say, will they take the same path, along the connective tissue fibers? Can fluid be demonstrated in normal connective tissue by means of highly diffusible dyes? Will mechanical forces which rub or squeeze the fibers together, enhance the spread of dye in the tissues? These questions were answered by experiments now to be detailed.

Methods

The methods employed for the present work were like those described in the preceding paper. Besides the vital dye pontamine sky blue a more diffusible dye, patent blue V was used. The nature of this dye and its preparation will be discussed below. In addition to the vital dyes two indicator dyes, brom thymol blue and brom cresol purple, were chosen. These indicators differ profoundly from the vital dyes in chemical constitution; they are far more diffusible than the latter and further are highly irritating to the tissues when used in strength, eliciting a prompt and marked edema. They were chosen because of the pH range of their color change. Properly adjusted they can change color within the tissues from blue or purple to buff. The thought suggested itself that these dyes, injected into the lymphatics and escaping through the tissues would practically disappear in the latter after changing to a buff color, but not before their irritant action had changed the fluid content of the normal tissue inducing an edema. At this stage dye in the blue form could be reinjected into the same lymphatics and its passage through the changed tissues observed. To obtain the indicators in such a form that they were capable of changing color, from purple or blue to buff, within either the lymphatics or the tissues, they were made up in 4 per cent aqueous solutions. These solutions, as shown by freezing point determinations with the Beekman apparatus, were isotonic with blood. Sufficient N/10 NaOH was then added to bring the pH of the brom cresol purple solution to 6.3 and that of the brom thymol blue to 7.0, with result that both were approximately 50 per cent dissociated. Very little NaOH was required. The solutions were injected intradermally into the ears of the anesthetized mice in the usual manner (1) and appeared promptly in the lymphatics.

The Effects of Free Interstitial Fluid upon the Movement of Dye through the Tissues.—As described in the previous paper, the vital dye pontamine sky blue after escaping from the lymphatics into the tissue, called out free fluid into the latter within a few minutes. The bristly colored projections of escaping dye disappeared and the diffuse blue color that took their place could be moved about at will by micro probes. The influence of the presence of free fluid in the tissues was much better shown by the experiments done with the two indicator dyes.

A description of some typical findings with brom thymol blue will suffice. In less than 1 minute after injecting it as a blue fluid into the lymphatics typical blue hair-like projections appeared outside the channels. They maintained their contours even when bent and twisted by the micro probe. Almost at once, however, diffusion from them began and within a minute and a half after dye first appeared outside the lymphatics, the colored projections showed the "second phase" of dye escape (1), that is to say, diffuse staining between them which could not be dislodged by the micro probe. Within another 2 or 3 minutes the color could easily be dislodged and the hair-like projections were scarcely visible. The irritant indicator had rapidly produced a local edema, as shown by the demonstration of free fluid. In another minute and a half, or even less, the diffuse blue coloration turned to buff. The color within the channels changed as well,—a phenomenon to be taken up in future studies.

In 44 experiments the lymph channels were gently reinjected with more of the indicator in the blue form after the change to buff had taken place outside, and now within a few seconds dye passed through the channel wall to form an evenly colored blue band outside it, with little or no indication of the hair-like projections as the dye spread outward through the tissue. Later the band turned buff. Pressure with the micro probe, exerted at once after the appearance of the blue color outside the channels, left a clear, colorless spot showing that the dye had been dissolved in the free fluid already present in the tissues which was squeezed away by the pressure. This occurred even before the blue band turned to buff. The free fluid induced by the previous injection had completely changed the state of affairs as concerned the movement of the dye. The experiments excluded all possibility that the appearance of the bristly and wavy colored projections, following dye injection into lymphatics, were due to the very slight pressure exerted when introducing the fluid. For dye was reinjected into the lymphatics at the same pressure in all of the experiments but its manner of transport through the tissues differed.

In the experiments described in the preceding paper the vital dye pontamine sky blue escaped from the lymphatics into tissue previously normal. The hair-like projections of color, seen under these circumstances, were also observed when the indicators first passed from the lymphatics into normal tissue. That is to say, these dyes, far more diffusible than pontamine sky blue and of a greatly different chemical constitution, seemed to move, like it, along the connective tissue fibers of normal tissues. When the irritant effect of the indicators had called out free fluid into the tissues in demonstrable amounts and the indicators were reinjected into the lymphatics the bristles of color were no longer observed, as the dye, escaping from the channels, moved outward through the tissues. Clearly a change in the state of the tissues had occurred which modified the manner in which the indicator dyes moved through them.

The same phenomenon was observed in eight experiments in which 2 or 10.8 per cent solutions of pontamine sky blue were introduced into lymphatics previously injected with one of the indicators. In these tests sufficient time was given to permit the escape of the indicator into the tissues, and its color change there before the pontamine sky blue was injected. Under these conditions the vital dye, escaping from the lymphatics, appeared in the tissues as a diffuse blue cloud: no bristles of color were seen. The findings indicate that the changed state of the tissues, in which fluid had accumulated, affected the mode of transport of pontamine sky blue and the indicator dyes, of widely different constitution and diffusibility.

Evidence on the Condition of Fluid in the Intradermal Tissues.—Although, in the previous work (1), we had been unable to demonstrate the presence of free fluid in the normal tissues of the mouse ear, the possibility remained that it was present there in amounts which defy detection. The diffusion of pontamine sky blue, escaping from the lymphatics, might have been so slow that it failed to reach the free fluid in the tissues before its own irritant action had called forth an edema.

To throw light on this point experiments like those described in the preceding paper were made with another dye, patent blue V,[1] which is much more diffusible than pontamine sky blue and like it tolerated in large doses when given intravenously, and calling forth a mild edema in about the same time. It was assumed that, being far more diffusible than pontamine sky blue, patent blue V should demonstrate sooner the presence of any free fluid within the tissues.

Patent blue V[1] has been used by us in many previous studies of the physiology of the lymphatics of mice, rabbits, and human beings (2–6). Like pontamine sky blue it is an acid vital dye and does not stain the formed elements of the tissue, during the periods required for the experiments. It is much more diffusible than pontamine sky blue having a molecular weight of about 585 (7).

Preparation of Solutions of Patent Blue V.—The dye was obtained in solid form as a crude technical dye, soluble to about 1 per cent in water and toxic to animals. It was converted to a more soluble, relatively non-toxic sodium compound. A weighed quantity of dye was suspended in distilled water and heated on a water bath. N/1 NaOH was slowly added with constant stirring till almost all the dye had gone into solution. The reaction started at once and one could perceive readily when almost all the dye had dissolved. The suspension of dye had a fluorescent appearance and the dissolved one a beautifully clear blue color. The calculated amount of N/1 NaOH to be added, approximately 150 cc. for each 100 gm. of dye, was not employed; instead we added only 80 to 130 cc., for the dye was not in pure form. (This precaution was taken to avoid an excess of NaOH.) After heating the mixture for ½ hour more, it was cooled, filtered twice and evaporated to a concentrated solution. The latter was dried in an oven *in vacuo* at less than 70°C. The dried material was then extracted three times with absolute alcohol. The latter when evaporated to dryness gave a good yield of non-toxic dye.

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[1] General Dyestuffs Company.

The preparation of the dye seemed to be better when small batches of the crude material were used. As determined by the freezing point method, aqueous solutions, 11 gm. in 100 cc. of water, were isotonic with blood.

The procedure as outlined above was employed to prepare the dye solutions used in this and in our preceding work. More recently a batch of dye has been received which, in the crude form, is more soluble in water than that obtained previously. Purification of the latest batch was carried out as follows: The dye was mixed in a mortar with dry, anhydrous sodium carbonate, 10 parts dye to 1 of the carbonate. Enough distilled water was added to make a paste and the mixture ground for 5 minutes. A little more water was added and the paste ground again for 5 minutes. Enough absolute alcohol was then poured on to make a final 95 per cent alcohol mixture; that is to say, the water previously added representing 1 part, 19 parts of absolute alcohol were added. After shaking in a mechanical shaker 20 minutes, the alcohol was filtered off through two layers of paper. The alcoholic solution evaporated to dryness yielded seemingly pure dye. This dye has not yet been used for biological experiments and it is suggested that others, employing patent blue V, may find other methods of purification necessary.

Aqueous, 11 per cent, solutions of patent blue V, isotonic with blood, were made up, and subsequently diluted with equal parts of Tyrode's solution or Locke's solution. The resulting 5.5 per cent solution had a tinctorial value about like that of a 2 per cent solution of pontamine blue and elicited edema in the tissues in about the same time. It was in approximately equimolecular concentration with a 10.8 per cent solution of the latter.

Observations like those described in the preceding paper were now made with both the diffusible patent blue V and the indiffusible pontamine sky blue. In escaping from the lymphatics and moving through the tissues both dyes showed the same sequence of events, already described as observed with pontamine sky blue. As will be seen below, only some of these events, but not all of them, occurred in shorter time when the more diffusible dye was used.

The 5.5 per cent solution of patent blue V, prepared as described, was injected into the lymphatics of the ears of 60 mice. With a stop watch the intervals were recorded at which the bristly colored hairs first appeared as also that at which they were first seen to broaden. In addition the time was taken at which a homogeneous blue color appeared between the bristles but could not be dislodged by prodding (the second phase), and the moment was recorded when the color was first found freely movable in the tissues, indicating the presence of free fluid in demonstrable amounts.

The lymphatics in the ears of 20 mice were injected with a 2 per cent solution of pontamine sky blue, and its movement through the tissues watched in the same manner. This dye solution possessed about the same tinctorial value as the patent blue V solution and the experiments served as controls.

We wish to stress at the outset that the judgments required for an experiment of the nature described were difficult and liable to subjective error. Yet in the absence of a better way to proceed, we feel the results to be worthy of consideration. They have been summarized in Table I, columns 2 and 4, together with the findings from another experiment, yet to be described. The table shows, in the appropriate places, the times at which the various phenomena were first observed in the tissues of the mid-third of the injected ears. It is to be noted that the various phenomena were often coexistent in any one ear, for example, in certain portions of an ear the second phase of dye escape might be seen while free fluid, a later event in the sequence, could be demonstrated elsewhere. This will be evident from the figures in the table.

The highly diffusible dye, patent blue V, escaped from the lymphatics rapidly. The bristly or wavy projections of color became wide sooner than in the control experiments, no doubt because the highly diffusible dye moved away from the bristles more rapidly than did the poorly diffusible one. Very shortly after injection one could see homogeneous coloration between the blue bristles, which resisted displacement by pressure; there existed in other words the second phase of dye escape. But freely movable color, indicating the presence of free fluid, did not appear much sooner than in the controls with pontamine sky blue. This observation indicates that there was no demonstrable amount of free fluid in the normal tissue which had remained unrecognized in the control experiments, and in our earlier tests, too, because of the indiffusibility of the dye pontamine sky blue.

TABLE I

The Time at Which the Phenomena of Dye Escape Occur, as Influenced by an Irritant in the Dye Solution and by Diffusibility of the Dye

	Poorly diffusible dye (control experiments)	Poorly diffusible dye (after addition of irritant)	Readily diffusible dye (no irritant)
Injected fluids	2 per cent pontamine sky blue	2 per cent pontamine sky blue plus ammonia water	Patent blue V
	min.	*min.*	*min.*
First appearance of colored bristles	Average: 4½ Extremes: 2–7	2 1–3	1¾ ¾–3
First definite widening of colored bristles	Average: 7½ Extremes: 5–10	4½ 3–7	3¼ 3 –4¾*
Second phase (first appearance of diffuse color between bristles; color cannot be dislodged by pressure)	Average: 7¾–13⅓ Extremes: 5–15†	In only one of 20 instances could this phase be identified	4 –7½ 3⅓–13‡
Free fluid (first demonstration of freely movable color)	Average: 10½ Extremes: 8–14½	5⅓ 2¾–9½	9¼ 8 –10§

The data of the table are explained in the text. In the control experiments (second column) the sequence of events took place more slowly than in tests in which 10.8 per cent dye solutions were used (Table I of the preceding paper).

* The colored projections became wide in a shorter time after injection than in the other experiments because of the diffusibility of patent blue V.

† This phase endures in some portions of the ear after free fluid has made its appearance elsewhere.

‡ The homogeneous coloration between the bristles was not displaced by pressure and remained just as long as in the controls.

§ Free fluid not demonstrated sooner than in the controls although the dye was more diffusible.

Another experiment was made to test the point. A tissue irritant was added to the solution of relatively indiffusible dye, pontamine sky blue, to bring free fluid rapidly into the tissues after injection of the mixture. We wished to see whether or not free fluid appearing in the tissues sooner than in the control experiments, would also be demonstrated sooner by the relatively indiffusible dye.

Varying amounts of C.P. ammonia water were added to the 2 per cent solution of pontamine sky blue in Locke's solution and injected into the lymphatics of the mouse ear in the usual manner. By trial and error it was found that the addition of 0.005 cc. of the ammonia water to each cc. of the dye solution brought demonstrable amounts of free fluid into the tissues rapidly. Intradermal injections of this mixture were made into the ears of 20 mice and its movement through the tissues watched, as first described, and compared with the control experiments.

The findings are summarized in Table I, column 3. When the ammonia in this experiment, acting as an irritant, brought fluid rapidly into the tissues, the poorly diffusible pontamine blue called attention to the presence of this fluid much sooner than in the control experiments. From this it was plain that the time required by pontamine sky blue to disclose the presence of fluid, in the preceding experiments, was not due to slow diffusion of the dye into fluid already present in the tissues, but to one of the two other possibilities discussed above: either free fluid was present in the normal tissues in amounts too small for dye dissolved in it to be visible, or else it was lacking.

The Spread of Dye through Tissue as Influenced by Slight Intermittent Changes in Pressure

The findings so far described in this and in the preceding paper suggest that connective tissue fibers play, indirectly, an important part in the transport of certain substances through resting tissues. Dyes of differing diffusibility and molecular size seem to move through tissues along or next to the fibers, but not as a diffusely colored cloud. The fact that free fluid of the connective tissues is present only in small amounts, or is lacking adds to the importance of the fibers as a pathway of interstitial transport. This being so, changes in pressure which rub or squeeze the fibers together should spread dyes more rapidly through the tissues. The mechanical effects of pulsation of blood vessels within a tissue increases the movement of fluid through it, as shown by increased lymph formation (8), and enhances the spread of dye as well (6). That slight changes in external pressure increase the interstitial spread of dyes has also been shown (5).

Attempts were next made, under high magnification, to find out what really happens when dye spreads through tissues subjected to slight intermittent changes in external pressure.

A tambour was constructed, as described elsewhere (5). After injecting the lymphatics with pontamine sky blue in the usual way, the ears of mice were placed over the tambour, between it and a glass cover slip for observations under the microscope.

After the bristly projections of color extending from the lymphatics became visible, intermittent pressure, in controlled amount, equivalent to a column of water 2 to 8 cm. in height, was brought to bear upon the ear by the tambour. Pressure was exerted for 1 second, with a period of relaxation of 2 seconds.

With each squeeze by the tambour the closely interweaving projections of dye could be seen bending, twisting, and bearing upon one another. They extended outwards with great rapidity and yet when the pulsations of the tambour were stopped and pressure was made upon them with the micro probe, the color could not be dislodged. This state of affairs lasted during only a minute or so of the intermittent pressure. Then a sequence of events took place like that already described in the preceding experiments, but more rapidly; the contour of the hair-like projections became less clear and free fluid became demonstrable in the usual way.

After more than one minute of intermittent pressure, the colored projections of dye could no longer be seen; and now when pressure was discontinued and the tissue prodded, the color was found freely movable, demonstrating the presence of free fluid.

The experiments showed that mechanical pressure, like that of massage, but far more mild, not only increased the rate at which dye spread through the tissues, but spread it through them in bristly or wavy colored lines. The path of dye movement seemed to be along or between the connective tissue fibers. The combined action of the dye and of the pressure changes brought free fluid into the tissues sooner than usual.

The Interstitial Movement of Dye Merely Brought in Contact with Connective Tissues

In the experiments so far described the passage of dye through the tissues was studied only after its escape from lymphatics while it moved in a direction opposite to that presumably taken by the fluids which form lymph. What can be said about the movement of dye that is not injected but merely brought in contact with connective tissue?

A method, described in an earlier paper (5) was available by which minute portions (0.0001 to 0.00005 cc.) of the isotonic 2 per cent solutions of pontamine sky blue were brought into contact with the tissues, through micro pipettes inserted into tiny micro puncture wounds. The fluids passed in by capillarity, no pressure being exerted, and formed little spots of dye, micro maculae as we have termed them (5, 6). Scores of micro maculae were made and their margins observed at high magnifications. Only a few colored hair-like projections were ever seen at the margins of these spots and then only for the first minute or so after the dye was instilled.

The advancing edge of color had a very different appearance from that of dye escaping from a lymphatic. The reason for this was not far to seek. In the experiments in which the lymphatics were injected, minute amounts of dye passed slowly into the tissues, accompanied by very minute amounts of water at most. When micro maculae were made the dye was present in a relatively large amount of free fluid, a state of affairs like that when it was present in edema fluid. Further, the dye itself causes edema, so that a local collection of edema fluid occurred at the margins of the maculae. Under such circumstances no bristles of color were to be seen, nor could they be expected to form. The presence of free fluid was demonstrable at all times by displacing the color with micro probes.

In other experiments of the same sort the intermittent pressures were brought to bear after dye maculae had been introduced into the tissues in the manner described. As already stated, their margins showed no signs of bristling, hair-like projections of color. But when pressure was exerted intermittently upon them, blue, hair-like projections suddenly made their appearance and were then seen to bend and rub together with each pressure change. As in the experiment just described, the blue projections elongated with great rapidity, while retaining their contours. But in less than a minute they faded from view, the color having become a displaceable cloud. If the intermittent pressure was stopped sooner, that is to say before the hair-like projections disappeared, this secondary change took place later.

These experiments with the tambour indicate that the rôle of the connective tissue fibers, in promoting dye spread, is even greater in tissues subjected to changing external pressure than in resting tissues.

DISCUSSION

The findings reported here confirm and extend those given in the preceding paper. There it was shown that the vital dye, pontamine sky blue, moves interstitially through the connective tissue of the mouse ear along or between the connective tissue fibers. Here it has been demonstrated that other dyes, differing from pontamine sky blue in chemical constitution and in diffusibility, apparently move through the normal tissues of the ear in a similar manner, that is to say along or between the connective tissue fibers. Dye, spreading through the ear more rapidly than usual, under the stress of changing external pressures, also seems to move along or between the fibers as they are bent and squeezed upon one another by the pressure.

Experiments designed to demonstrate the presence of free interstitial fluid have failed. This being so, the function of connective tissue fibers as pathways of interstitial transport becomes of greater importance.

What can one infer about the movement of other substances or of water through the tissue? What does our work show about the state of interstitial fluid or about the nature of the interstitial spaces? Do the latter indeed exist? To discuss these questions it will be necessary to recall from time to time evidence which has been presented recently in several papers from this laboratory.

First it may be recalled that pontamine sky blue (mol. wt. about 990) and patent blue V (mol. wt. about 585) are acid vital dyes. They do not become fixed upon the formed elements of the tissues during short periods of time such as were employed in our experiments. One of the experiments reported here showed that the diffusible dye spread more rapidly through the tissues of the mouse ear than the indiffusible one, a fact supporting the view that their spread through the tissues is probably like that of other molecules of the same size and of similar physicochemical constitution.

From the behavior of the dyes we can make no attempt to judge of the movement of fluids; it may be faster or slower than that of the colored particles or even in the opposite direction. For example, we cannot say whether or not fluid moved outwards from the lymphatics as the dye escaped from them. On the other hand, we can judge from the behavior of the particles whether or not there is more or less water in the tissues and whether the water can move freely in them. This point need not be labored here—it has been amply discussed in the body of the paper. The presence of visible amounts of fluid stained blue by the dye could be recognized by exerting pressure on the tissues with the micro probe, the colored fluid moving interstitially as result.

But why do dyes travel along or between fibers? Are they adsorbed on them? Is there an interstitial space about each fiber,—a crack, so to speak,—between it and an interstitial ground substance? Is there a relatively thick layer of water about the fibers or is there a thin film? Or is there merely an interface between two adjacent surfaces? Adsorption of dye, or of other substances of large molecule, upon the fibers will not explain all the observed phenomena. If it occurs, it must enhance the rôle of the fibers in the transport of substances through tissues, as will be seen below.

It is universally agreed that the appearance of tissues in fixed microscopic sections is misleading. The spaces which appear between the formed elements are produced chiefly by shrinkage in the process of dehydration. All workers using methods of microinjection have been struck by the fact that the connective tissues resist the introduction of fluids under pressure and behave as though the cells and connective tissue fibers were imbedded in a continuous ground substance. In this laboratory we have frequently observed that the tissues of the ear of the mouse offer a strong resistance to the injection of dye solutions through a small pipette (0.5 mm. in external diameter and about 0.2 mm. in bore). Fluid under slight pressure does not enter from the pipette until the latter is partially withdrawn, leaving a space previously occupied by its shaft. Dye solution then rushes at once from the pipette to fill this space, but as soon as this has happened resistance is encountered again. If the pipette is once more partially withdrawn, more dye promptly escapes into the newly formed space. As result there appears in the tissue a dye-stained track looking like that obtained when a wire dipped in dye solution is thrust into a block of gelatin or agar and rapidly withdrawn. The concept of wide tissue spaces like those seen in microscopic sections, but filled with free fluid, is inadequate to explain these phenomena.

During the present work suggestive evidence has been found of the presence of a matrix in the interstices between the formed elements. The possibility of the existence of such a matrix has been fully discussed by others (9–13). In most of our experiments dye, after appearing as discrete, hair-like projections between or upon the connective tissue fibers, spread from them and colored the neighboring tissues a diffuse blue. The fact that pressure over these diffuse blue areas failed to squeeze away the color showed that it was not dissolved in free fluid. When pressure was brought to bear the paling in color that took place was like that seen *in vitro*, under the microscope, when blocks of agar containing the dye are pressed upon, and in this way thinned. The change was seen in the ears whether diffusible or indiffusible dye was used. Furthermore the interval elapsing between the time of injection and the moment at which colored free fluid became demonstrable was the same whether the diffusible or indiffusible dyes had been employed. Had free fluid been already present in the tissue, it would have become demonstrable sooner with the highly diffusible dye capable of moving into it more rapidly. An irritant, ammonia, called out fluid so quickly that the stage of diffuse staining, not due to free fluid, the second phase, so called, was not recognizable. From this observation we can conclude that the diffuse blue coloration with no demonstrable free fluid, to be noted under ordinary circumstances, was due to dye distributed through some sort of matrix which could not be displaced by pressure.

It is conceivable that our method is adequate to demonstrate fluid only when edema exists. Since the method can and does demonstrate microscopic amounts of fluid, the objection, if valid, serves to emphasize a point we wish to bring out in these papers, that at most only submicroscopic quantities of fluid can be free in the tissue. If interstitial pools of fluid actually exist, through which there is a movement of fluid, they must be far smaller than the usual histological section would lead one to believe, so small as not to be demonstrable on sensitive test with a dye. How does fluid move through tissues if not through spaces? The rôle of connective tissue as a pathway for the spread of dyes has been stressed. It is possible that fluid movement through tissues may take place between or surrounding connective tissue fibers and cells like the dye movement, perhaps in thin films, so thin that the water or fluid is no longer free in the usual meaning of the word, but is held to the fiber firmly by surface forces. Something of this sort will account for the finding that dye cannot be dislodged by pressure from its position along or between the fibers until its presence has called forth excess fluid, that is to say an edema. The state of tissue fluid may very well be analogous to that of a film of water caught between two pieces of glass, to all purposes captured, unable to move freely this way or that, but still chemically capable of behaving as fluid,—to diffuse into cells, to transport ions, to permit the exchange of solutes through it. The term "captured" has been used to avoid the concept of chemically "bound" water. The captured films of water, if they exist, must be so thin that they are practically a part of the connective tissue, not interstitial pools of fluid. This concept of fluid captured by capillary forces would explain the fact that fluid does not normally seep through the tissues and collect in the dependent portions of the limbs.

It is almost needless to point out that these remarks do not in any way contradict the known fact that 20 to 30 per cent of the body fluid is extravascular and extracellular, as shown by the work of Lavietes, Bourdillon, and Klinghoffer (14) on the distribution of sulfocyanate between the blood and tissues; by the work of Bourdillon and Lavietes (15) on the distribution of sulfate; and by that of Harrison, Darrow, and Yannet (16), Hastings and Eichelberger (17), and others (18). Our findings, here reported, yield no information on the amount of fluid in normal tissues, they indicate only that the interstitial fluid is not freely movable fluid lying in pools or lacunae, the so called tissue spaces.

It is to be noted that our studies have been made on the connective tissue of the ear of the mouse. In this tissue the presence of a large amount of extravascular, extracellular fluid can be accounted for by its existence on surfaces. As the diagrams and photographs of the preceding paper indicate poorly, the colored projections we observed are extremely numerous, constituting a fuzz on every lymphatic capillary. The connective tissue fibers about blood capillaries are equally numerous (19). The vascularization of the connective tissue of the mouse ear is exceedingly rich (20). It is important for the purposes of this discussion that the reader should inspect Fig. 10

of the article just referred to. Only from the photograph there shown can one appreciate the richness of the capillary plexuses of the ear. The millions of fibers attached to vessels in the connective tissue must present a great surface area. To what extent our conclusions are applicable to other, less vascular tissues, cannot be said at present.

We are not alone in the belief that there is no substantial amount of free interstitial fluid, to use the word "free" in the sense in which it is usually understood. Clark and Clark (21), in studies of the blood capillaries in the tails of amphibia, and in transparent chambers in the ears of rabbits, have noted that particles and cells present in the tissues outside the vessels show no brownian movement and they infer that normally there is no free fluid present. Occasionally, in states of inflammation, they have observed spaces existing about blood vessels which sometimes contained free fluid as shown by the brownian movement of particles in it.

One other point deserves mention. Earlier work from this laboratory (22, 2) has shown that blebs of dye solution resulting from the forcible injection of the colored fluid into the ear of the mouse spread chiefly from the periphery of the ear toward its base. The dye maculae of the present work also spread in this manner although they were not under pressure. This spread of the dye toward the ear base occurred in ears lying horizontally upon porcelain placques and cannot be attributed to gravity. Recently Peters (23) has referred to the work as evidence for the presence of free fluid in the tissues under normal circumstances. Formerly we too believed this to be the case, but our later studies have shown that the dyes we used elicit an edema, the condition being very different from the normal as the present work sufficiently attests.

The phenomena here reported offer an explanation for many of the findings of our previous studies (5, 6, 8) on the factors governing the spread of substances through tissues. These have shown that mechanical forces have major importance in promoting the interstitial spread of dyes and of fluids. Slight changes in external pressure yielded the most rapid interstitial spread of dye that we have ever observed (5). In quiet, resting tissue, through which dye moves in hair-like projections, the mechanical effect of the pulse increases the interstitial spread of dye (6). It also increases the formation and flow of lymph (8), indicating that the movement of fluid through the tissues must be increased as well as the movement of dye. The appearance of edema in tissues perfused with a non-pulsating current of blood does not lead to an increased spread of dye (6) but if a pulsating flow is used dye spread is increased. In normal tissues dye spread is greater than in edematous tissue (5). In hyperemic normal tissue becoming edematous, but not yet boggy, dye spread is still greater. Large amounts of free fluid in the tissues fail to increase dye spread.

Dye spread is greatest at those times at which one can observe the extension of colored projections of dye along or between the formed elements of the tissues. Our studies on the mechanical effects of the pulse and on changes in external pressure indicate that substances move most rapidly through the tissues while the formed elements are close together and not separated appreciably by edema fluid. At such times the periodic changes in caliber of the vessels, brought about by the pulse, or the mechanical effects of changes in external pressure seem to increase the interstitial spread of dyes by squeezing together or weaving the fibers and formed elements of the tissues. It has just been mentioned that in normal hyperemic tissues not as yet demonstrably edematous but on the way to becoming so, dye spread is greatly increased (5). So too is the formation and flow of lymph (6, 8). These findings indicate that the movement of fluid from the blood through the tissues to the lymph is also enhanced at those times at which the formed elements are not appreciably separated by edema fluid. From this it would seem that the movement of the dye is probably increased by that of the fluid. This is further shown by the fact that the perfusion of edematous tissue (6) by large volumes of blood at high, unvaried pressure leads to but little dye spread and to little formation or flow of lymph (8).

It is conceivable that a perifibrillar movement of substances may be the method of supply of nutriment to tendons, structures which are notably avascular. One may even hazard a guess that the transport of nutritive substances for the central nervous system takes place to a large extent along fibers. It has long been recognized that in nervous tissue the vascular feet of the astrocytes form the connection between capillary walls and the interstitial tissue of the central nervous system (24). Further, as shown in some of the photographs in the previous paper we have seen dye, after its escape from the lymphatics, spread out along the sheaths of peripheral nerves. These and other problems must wait for future work. Whether dyes escape from blood capillaries along fibers is now under investigation.

SUMMARY

The interstitial movement of several dyes of widely different chemical constitution and diffusibility, in the connective tissues of the mouse ear, has been observed at high magnification. Dye extension seems to be conditioned by the form and structure of the connective tissue fibers. After escaping from the lymphatics of the ears of living mice, each dye appeared first in the tissues as bristly projections of color. These bent and twisted when pressed upon by a micro probe but sprang back into place when the pressure was removed. The present work and the preceding have shown that the lines of color are formed by dye along or between connective tissue fibers.

Intermittent external pressure applied to the tissue, squeezes and bends the fibers together and greatly increases the spread of dye along them. The connective tissue fibers assume an important rôle in the spread of substances through tissues subjected to pressure changes.

The experiments have given evidence of the existence of a tissue matrix in the organ but none of the presence of free interstitial fluid. In tissue subjected to irritant stimuli and in frankly edematous tissue, free fluid is readily demonstrated. When it is present the method of extension of dye is completely changed. Dye appears in the tissues as a diffusely colored cloud which can be freely moved by pressure with a micro probe.

The bearing of this evidence upon the condition of interstitial fluid and the nature of the interstitial spaces is discussed.

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A COMPARISON OF VIRUS-INDUCED RABBIT TUMORS WITH THE TUMORS OF UNKNOWN CAUSE ELICITED BY TARRING

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PLATES 20 TO 27

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The agents called "carcinogenic" act by inducing a protracted tissue disturbance, on the basis of which discrete tumors of unknown cause arise. We have encountered many such growths during experiments on the action of tar to alter rabbit skin in such a way that cancers develop (1) after infection of it with a virus which causes only benign papillomas ordinarily (2). A knowledge of the tar tumors proved essential to appraisal of this phenomenon and hence their study was undertaken. It disclosed the fact that in rabbits such as we employed the benign tumors elicited by tar were of two sorts only, and these so definite in type as to imply the action of specific causes. The commonest sort were papillomas differing distinctively from virus papillomas in certain cytological details but closely resembling them in cellular derivation, mode of development, general morphology, behavior, and fate. The results of a point by point comparison made it plain that the unknown cause of the tar papillomas had neoplastic effects like those due to an authentic virus. The present paper is concerned with all of these subjects.

Material and Methods

Our rabbits, procured from New York dealers, were hybrids of the common, brown-gray (agouti) type. The tar came from the Ostergasfabrik of Amsterdam, and was the generous gift of Dr. Landsteiner. It was smeared on the inside of the ears twice a week, for from 2 to 4 months in most instances, with stripping of the accumulated layer at every third tarring. Over one hundred animals were tarred, mostly as controls in experiments with virus, and some were kept for many later months and sometimes tarred again.

Occasionally the tarring caused tumors to appear within the first month, and they did so within 3 months in more than half of the animals. They were studied by preference soon after they arose, when only a few millimeters broad, before they had undergone the secondary alterations in structure that are frequent when tarring is kept up. Numerous biopsies were made, with a sharp cork-borer. All the material was fixed at once in acid Zenker solution, since in specimens from animals dead only a few hours the finer cytological details underwent change. The stains were methylene blue and eosin.

The Kinds of Tar Tumors

Tar was the first substance utilized in purposeful carcinogenesis, and it was applied to rabbits; but attention soon shifted to mice as a more favorable material. The large literature has been several times reviewed (3), and we have recently summed up the gross effects of the tar employed in our animals (1). It caused benign tumors early and frequently, yet gave rise to cancer only after many months and then but seldom.

Microscopically the tumors fell into four well defined categories:

1. Common Papillomas.—Yamagiwa and Ichikawa (4) termed these growths stalked and broad-based folliculo-adenomas, and noted that the latter were the more aggressive, with carcinomas sometimes originating from them. Both sorts were very frequent in our animals (Table I); and they were found to have essentially the same cytological characters and to grade into each other. They were made up of stratified squamous epithelium, thick and obviously abnormal yet keratinizing in an orderly way, supported upon narrow connective tissue cores or fingers, which occasionally branched (Figs. 5, 7, 40). They first appeared as small, subepidermal mounds which later became discrete, superficial growths of conical, cauliflower, or onion shape, or else,—when maceration had occurred beneath the tar,—raw, fimbriated discs or hassocks. The keratinized epithelium was tenacious, often building high. When tarring was kept up the aggressive growths extended sideways and often downwards in blunt epithelial foldings and tongues, with result that they had a broad base. The shape of indolent ones depended on the changes occurring in the underlying connective tissue. When it proliferated greatly they sometimes became tangential, fleshy spheres, while through its secondary contraction they often became constricted at the base (Fig. 1) or stalked or pedunculated. Persisting broad-based growths eventually underwent similar changes, save in the occasional instances in which they became malignant. Papillomas that had been many times tarred often reached a diameter of several centimeters, and then as a rule they consisted almost entirely of connective tissue. Sometimes their weight gradually pulled them out into pendulum form after tarring was left off, and not a few were torn away while others contracted into mere tabs as result of sclerosis.

If tarring was left off soon after the papillomas appeared, most of them vanished rapidly. Those that were left dried down, often to mere scabs until reconstituted from beneath, and many lost the fleshy character, becoming horns or dry cauliflowers. They tended to persist in proportion to the general changes induced in the skin, and when these had been great, new papillomas often arose. If tarring had been kept up for many months, until they were numerous and crowded, maceration and bacterial infection often persisted later, furthering their enlargement, and occasionally the aural orifice became blocked with pultaceous matter and the animal died of sepsis. Large papillomas tended to persist because their core of sclerotic connective tissue could not be resolved; but sometimes their papillomatous epithelium was ultimately replaced by smooth, non-neoplastic epidermis.

TABLE I

Relative Frequency of the Benign Tar Tumors: Dependence of the Carcinoid Form upon Tarring

Group	Number of rabbits	Period of tarring	Further period after tarring	Papillomas	Transitional growths	Apparent carcinomas (carcinoids)	Frill horns	Total growths
		days	*days*	*number*	*number*	*number*	*number*	*number*
A	18	54–118	2–40	50	10	64	5	129
B	18	61–121	57–259	93	2	0	13	108

The growths tabulated came from groups of animals tarred for the same general length of time, but differing in that the tumors were not procured from one group until long after tarring had been stopped. Only growths which were examined microscopically are listed, and in some animals they formed but a small proportion of the total number. The basis of the classification is given in the text. The frill horns could be readily identified in the gross, and every one seen was taken for section. Carcinoids also were often recognizable as such, and a greater proportion of them were examined than of papillomas. It follows that frill horns were actually much rarer than the figures indicate, and carcinoids somewhat less common.

The papillomas have often been pictured. The several hundred that we have studied microscopically differed not a little in general pattern. Some were completely superficial and regular, with widely spaced papillae and dry keratin between; others had many crowded peaks; others extended down in broad tongues that keratinized secondarily, often becoming cystic; while others still broke up along the base into what appeared to be anaplastic, squamous cell carcinomas. Yet despite this diversity the cells of all, so long as they remained benign papillomas, manifested identical *qualitative* alterations (Figs. 33 and 35), and whenever differentiation and keratinization took place it was in the same way. The minute histological findings will be described further on.

2. *Frill Horns.*—Though possessed of a distinctive morphology, these growths have not heretofore been reported. They were seldom more than 3 mm. across at the base, yet were readily recognizable in the gross because they built up into narrow, cylindrical or strap-shaped, curved or twisted horns (Figs. 8, 9), often 2 to 4 cm. high, of the same diameter everywhere, usually cream-colored but sometimes light or dark gray, and always dry nearly to the skin level. Here was a slightly bulging, fleshy collar covered with epidermis stretched smooth.

The frill horns arose at the same time as the papillomas, but were infrequent (Table I). Usually there were several when any. Though appearing wholly superficial, they burrowed slowly, and ineffectually, their epithelium extending downwards in a jagged, crescentic frill (Figs. 10, 11), which advanced so gradually as to be effectively barred from the cartilage by reactive connective tissue proliferation. Very occasionally a fold in the frill mimicked a papillomatous finger. Its living layer was not so thick as in papillomas, and stained deeper with methylene blue. The individual cells were irregularly polygonal, with birdseye nuclei, and they became larger when differentiating, and keratinized abruptly without preliminary granulation or flattening (Fig. 12). The keratinized material stained pink or red with the eosin and methylene blue stain, whereas that of tar and virus papillomas failed to color or took on a faint blue; and the nuclei of the cells newly dead stippled the keratinized material with dark blue, oval dots, which faded later. In the gross the horns were close-textured, horizontally laminated, sometimes with an ill-defined, vertical striation, and microscopically their keratinized material appeared dense, showing none of the loose, reticular pattern, indicative of old cell boundaries, which is visible in papillomas. The connective tissue underneath the growths, like that under papillomas, showed only chronic inflammatory changes, with a scattering of macrophages and lymphocytes. Continued tarring had little effect to render the epithelium disorderly, and when it was discontinued the horn nearly always went on growing, unlike most papillomas, and sometimes new ones appeared (compare frequency in groups A and B of Table I). Retrogression was noted only once.

3. *Carcinoids.*—A large proportion of the tumors appearing early had the morphology of squamous cell carcinomas (Figs. 13, 14, 16, 18), yet when tarring was left off they vanished or assumed a benign form (Figs. 17, 19). These were the growths generally termed carcinoids (5). Often they were markedly invasive at first (Fig. 15), their cells straggling down individually, or penetrating in narrow strands through a reactive connective tissue which was sometimes mucoid like that elicited by many squamous cell carcinomas; and not infrequently they extended through lacunae in the cartilage to the outside of the ear, forming mounds there which ulcerated. They could usually be recognized in the gross, being raised, raw, ragged discs with infiltrating edges (though papillomas occasionally assume this form), or else raised, discoid ulcers as much as 1.5 to 2 cm. in diameter. Yet even when proliferating within lymphatics (Figs. 15, 18) they never metastasized; and though tarring was kept up they did not continue to grow destructively but eventually took on gross forms like those of old papillomas, or else retrogressed.

Repeated biopsies showed that the supporting connective tissue had proliferated actively, walling off the growths, and that in proportion as this happened their epithelium ceased to invade, underwent differentiation, and in most instances became papillomatous (Figs. 16, 17), though cell islands of carcinomatous aspect occasionally persisted. Frequently the deeper epithelium rounded up into cysts filled with keratinized material (Figs. 18, 19) and lined with a stratified squamous layer devoid of neoplastic features; but in other instances all the deep cells keratinized, died, and were eventually resorbed. Those carcinoids which disappeared did so in these ways, save in an unique instance of a growth dying *en masse* without differentiation, as if from some intercurrent malady. Carcinoids which appeared early, before the tarring had effected great cutaneous changes, generally vanished soon after it was left off, whereas when the skin had undergone much alteration they often persisted, either as papillomas or cysts.

The earlier observers, conceiving that the carcinoids were cancers, followed with intense interest the various retrogressive changes here described (6), and these have been many times figured. Some workers attributed them to the associated connective tissue alterations whereas others held that this could not explain them.

The papillomas of animals tarred 2 to 4 months not infrequently broke up along the base into what looked to be carcinomas (Table I, Transitional Growths). Yet when tarring was left off these all underwent one or another of the changes just described. The figures of Table I show that a wholesale disappearance of carcinoids took place then. Amongst 129 growths of 18 rabbits, examined 2 to 40 days after the last tarring, 64 had the morphology of squamous cell carcinomas and 10 were transitional growths. Among 108 tumors procured 61 to 121 days after the last tarring of 18 comparable animals, there was not a single apparent carcinoma and only 2 transitional growths. All the rest were either papillomas or frill horns. Keratinized cysts have not been included in the figures.

To determine whether the carcinoids would grow in a new situation, pieces of 20 that were big and actively enlarging were punched out, hashed separately in Tyrode solution, and implanted in the voluntary muscle or connective tissue of the axilla or groin of the host. Here they behaved as did bits of 19 tar papillomas treated in the same way, their cells either differentiating, dying, and disappearing without sign of growth, or rounding up into pearls lined with stratified squamous epithelium (7). Similar results had been reported by Ferrero (8). Hair follicle epithelium, sebaceous glands, and bits of cartilage, accidentally introduced with the implants, survived in the new situations.

4. Carcinomas.—In the early work on the tar cancers of rabbits (4, 6) carcinoids were frequently taken for carcinomas. Recent authors agree that usually the latter develop only after tarring has been done for a long time. Guldberg (9) elicited papillomas after 61 to 140 days in all of 23 rabbits, but carcinomas appeared in only 7 and not until the 276th day of tarring at the earliest, and in most cases at about the 330th day. Our tar produced nearly always a fatal toxemia or liver cirrhosis, when applied regularly for more than a few months, not sufficiently long to elicit cancers. These developed, however, in 2 animals tarred again after an interval. Tarring of one of them had been done throughout periods of 5½ and 6 months, the cancer appearing after 21 months in all. The first tarring of the other was for 4 months, with later applications for periods of 4 weeks and 3 weeks. Between the 20th and 22nd months 2 cancers appeared. In both rabbits metastasis took place to a regional lymph node.

Most tar cancers of rabbit skin are squamous cell carcinomas arising secondarily from papillomas (4, 6). The 3 we have studied had this derivation, and the metastasis of one was cystic and exhibited the papillomatous form (Fig. 20). The literature reports occasional trichoepitheliomas and carcinomas originating from sebaceous glands, as also spindle cell and polymorphous cell sarcomas.

Effects of Tarring the Tumors

In a previous paper we have dealt with the influence of continued tarring upon the morphology and behavior of virus-induced growths (10). It has even more pronounced effects on many of the tar tumors, and is often the determining factor in their fate.

Tarring causes acute inflammation of the skin, with chronic changes later if the applications are kept up (3). In some of our animals the ears were thick and hot when tumors first appeared, and not infrequently a foul maceration had taken place under the tar with result that the growths were exposed to bacterial infection, while additional opportunities for this were provided by trauma incidental to the repeated stripping of the tar. The conditions were such as are known to further the growth of tumors generally. In the loose, proliferating connective tissue most of the carcinoids grew fast and invasively; and it was frequently edematous, not only beneath them but sometimes opposite them on the outer side of the ear (Fig. 47). Thither they often extended through lacunae in the cartilaginous plate, sometimes by way of the large lymphatics. Ferrero (11) has noticed that connective tissue disturbances favor carcinoid activity. The papillomas also tended to grow rapidly and irregularly, where the connective tissue was most disturbed, and sometimes they became anaplastic along the base. The frill horns, on the other hand, proved unresponsive to stimulation, as has already been stated.

The continuance of tarring notably stimulated the activity of papillomas and carcinoids, and leaving it off had pronounced adverse effects upon them. Unless it had been long kept up the skin at once began to revert to the normal, the acute inflammation and edema disappearing, the circulation lessening, the connective tissue sclerosing, the epithelial proliferation slowing, and the epidermal surface drying down and desquamating. The tar tumors dried down too, and most of the papillomas disappeared, while the carcinoids vanished as well, or became papillomas or cysts. The more rapidly the skin returned to the normal the more sweeping was the disappearance of growths. In occasional animals tarring called forth tumors very early, in 4 to 5 weeks (Fig. 18), and sometimes then the ears were studded with them, many being anaplastic carcinoids as biopsies proved; yet if no more tarring was done all disappeared within a fortnight. In these cases the ears had undergone but slight general changes and rapidly resumed the normal appearance.

Very different was the outcome when tarring had been kept up 3 to 4 months or had been done throughout several periods. The ears then remained thickened and indurated for many weeks, and scurfy layers formed and reformed on them, attesting to persistent epithelial activity. That the other tissues were also in an abnormally excitable state was shown by the rapid and complete filling in of holes 3 to 10 mm. across which had resulted from punch biopsies. Even though the ears presented a normal aspect when the holes were made these closed more quickly than usual, sometimes with cartilaginous thickening, while when the skin had remained thickened and scurfy not only was healing notably rapid but there was often a lumpy overgrowth of cartilage. The conditions thus disclosed were obviously favorable to the tumors, as shown by their frequent persistence and increasing disorder, and by the appearance in some instances of new ones. They proved most likely to persist along the edges and near the tips of the ears where trauma acted to prolong the abnormal state of affairs (Fig. 1); and foci of inflammation beneath them or in their stroma frequently aided their course and acted to distort their form. Some of these findings are not new, yet all deserve emphasis because they bear upon the riddles presented by the occurrence and disappearance of the carcinoids, and the eventual change of papillomas to carcinomas.

The Place of the Carcinoids as Tumors

The carcinoids arose at the same time as the papillomas, and exhibited a like tendency to disappear after tarring had been left off. They often derived from papillomas and those persisting frequently became such, and were then indistinguishable from growths primarily papillomatous. Some of the carcinoids however retrogressed to keratinized cysts, as rarely happened with papillomas; but this was only because the latter did not penetrate deep. When they did so, or were implanted within the host, the cystic form was frequently assumed; and the cysts differed in no wise from those originating from carcinoids. There was obviously a close relationship between the two sorts of tumors. In seeking to understand it certain observations on virus papillomas come to mind. These growths often assume a malignant appearance if experimentally stimulated or implanted in notably favorable locations, especially if inflammation of bacterial origin ensues (12); and yet they are not true cancers, for they revert at length to the papillomatous state. We have termed the phenomenon *factitious malignancy*. Everything that is known of the tar carcinoids indicates that they are expressions of a factitious malignancy of growths which have intrinsically the nature of tar papillomas. Their complete dependence upon tarring for their cancerous form and behavior becomes comprehensible in this light, as does also their frequent derivation from papillomas and transformation into them, or into keratinized cysts such as papillomas may secondarily form.

Many carcinoids have a malignant aspect from the first (Fig. 13). Becoming visible as small, subepidermal mounds, they grow down from an intact surface epidermis or from the epithelial cells of hair follicles; and if tarring is stopped they may die by differentiation and keratinization, and be resorbed without giving sign of any change to papillomas. The question arises of whether growths of this sort, and those carcinoids of secondary origin which disappear in the same way, do not differ intrinsically from the ones which undergo conversion into papillomas or cysts. The answer is supplied by the course of events when tarring is kept up. Then the great majority of the carcinoids keep on proliferating, though only to assume the state of papillomas when tarring is eventually left off (group A, Table I), or, if disappearing, to undergo the same changes as these. It follows that such carcinoids as die by keratinization do not differ from the rest save in having even less ability to maintain themselves.

All in all the conclusion seems justified that the carcinoids owe their malignant aspect and behavior to the effect of extraneous conditions upon growths having an intrinsic character which ordinarily finds expression in benign papillomas.

The greater the acute inflammation of ears on which tar has evoked tumors the greater was the proportion of growths exhibiting the carcinoid form. Sometimes all showed it for the time being. Yet frequently two tumors situated side by side, exposed that is to say to much the same local conditions, were the one a carcinoid, the other a papilloma (Fig. 24). The inference seems justified that papillomas differ much in their responsiveness to those local influences which result in spurious malignancy.

It has been frequently assumed that the carcinoids are carcinomas brought into being and maintained by tarring, and devoid of the power to grow independently. Carcinoids differ, however, from true carcinomas, not alone in their inability to proliferate independently as such, but in a more significant feature, namely the reversible malignancy of their cells. Real squamous cell carcinomas of man have been known to retrogress on rare occasion,—and they do so by differentiating and keratinizing completely (13), as happens with many carcinoids,—but they never, like the latter, undergo conversion into benign growths. Carcinomas they remain until the last.

A few of the small tar tumors procured from animals which survived long tarring, or which were tarred throughout several periods, were found to have the microscopic aspect of cancers, although tarring had been discontinued for many months. The general state of the ears was notably pathological, and one may assume it to have given unusual encouragement to the persistence of carcinoids; yet the development of true cancer, if only in three instances, indicates that the apparent malignancy of some of the small growths mentioned may have been real.

Comparison of the Tar Papillomas with the Virus Papillomas

The resemblance of virus papillomas to the tar papillomas has already been reported upon briefly (14). The virus growths studied had been obtained by the direct inoculation of scarified normal skin, mostly of the animal's side, and in consequence they derived from the surface epithelium, and were subjected to none of the collateral influences that tarring exerts. Now we have studied many papillomas due to the localization of virus out of the blood stream into the skin of ears repeated tarred, or acutely inflamed by the application of carbon bisulfide, as also growths resulting from the direct infiltration of normal ears with the inoculum, by way of a vein, with tarring later. Tar has also been applied repeatedly to virus papillomas resulting from tattoo inoculations into normal ears. These diverse materials gave consistent findings. Previous experiments had demonstrated that when skin has been tarred for some weeks prior to infection with virus by way of the circulation a considerable variety of tumors results, some of them cancers (1). For the present comparison only those virus papillomas were utilized which arose under conditions excluding this complication, most of them from ears which had been tarred just enough to bring about localization of the virus, or from ears which were not tarred until after virus infection had taken place.

Many of the facts to be cited of the tar papillomas are truisms, and no reference will be made to the literature substantiating them. The pictures illustrating the comparison generally show growths which had not yet undergone the complications which result from continued tarring and inflammation of the supporting tissues. In other respects they are representative.

Cellular Derivation.—Tar papillomas derive mostly from the epithelium of hair follicles, whereas those produced by virus rubbed into scarified skin all spring from the surface epithelium (2), as just remarked. But if the virus is thrown into the blood stream after one or two tarrings of the ears to insure localization, most of the growths arise precisely where the tar tumors begin, namely at the lower side of the hair follicles (Figs. 21, 22). This is the case also when normal ears are directly infiltrated with virus fluid by way of a marginal vein, and are tarred a few times later to render the inoculum effective (10). As in the case of tar papillomas the growths arising from the follicles usually begin on their basal side, with a proliferation of cells in the stratum germinativum, resulting in the formation of blunt, broad processes ("elephant's feet"), and a folding inwards of the epithelium in the papillomatous pattern.

Morphology.—On the inside of the ear both the tar and virus papillomas soon erupt and become surface growths. On the outside, though, where the skin is not bound tight to the cartilage, they may reach a diameter of 4 to 10 mm. before the stretched, shiny epidermis over them dries or gives way. Here they lie embedded as more or less flattened acorns or spheres (Fig. 18 *b* of reference 15). Most of those due to tarring are creamy or pink, though some are light gray and a few dark; whereas the virus papillomas are much more frequently pigmented, often dark gray or almost black. Both are opaque toward the center, and here they consist of keratinized material formed by a proliferating rind of stratified squamous epithelium of abnormal character (Figs. 23 to 26). This is usually thickest where deepest; and its infoldings are usually somewhat more numerous and crowded in the virus papillomas (Figs. 27 to 30),—as would follow from their greater vigor. The pattern becomes more complex as the growths enlarge, and the epithelium may thrust irregular tongues downwards (Figs. 29, 30), an invasion encouraged by continued tarring.

After they have erupted the papillomas of both sorts become superficial cones, cylinders, "cauliflowers," or "onions," or they form tall, dry horns. Their keratinized material is tenacious and builds high. They may consist of it practically to the base, or connective tissue processes covered with living epithelium may extend far up into it. The processes branch sparsely (Figs. 4 to 7). As a rule virus papillomas proliferate the more rapidly and hence are plumper, more fleshy, do not dry so far down, or split so frequently into cauliflowers, retaining instead the onion or conical shape (Figs. 2, 3). Yet when tar papillomas grow rapidly, or virus papillomas slowly on the same ear, they cannot be told apart individually in the gross unless they are melanotic, which is never the case with actively enlarging tar papillomas though frequent with those due to virus (15).

Under low magnifications the tar and virus papillomas are nearly alike (Plate 20 *et seq.*), but they differ distinctively in their finer cytology (Plate 25). In the virus growths the proliferating cells of the stratum germinativum are larger than normal, with abnormally big, vesicular nuclei (Figs. 32, 34). They get larger still as they differentiate, undergo but little flattening in the stratum granulosum, and the granules forming there are usually few, and range from small to very coarse. The cells of the tar tumors on the other hand, though unusually large in the stratum germinativum, do not increase further in size, and they flatten in the stratum granulosum, appearing fusiform or oat-shaped on cross section, with numerous small granules darkening the cytoplasm (Figs. 33, 35). The nuclei of the virus papillomas, big and vesicular from the first, increase markedly in size as differentiation goes on, and the chromatin marginates, whereas in the tar papillomas they alter little, though large primarily, and the chromatin remains central, giving a birdseye appearance. On keratinization the nuclei of the virus growths sometimes become pycnotic for a brief while, in the deep crypts between the papillae (Fig. 32), whereas those of the tar papillomas usually lose almost at once their capacity to stain. The keratinized scales, however, assume the same appearance in both instances, their outlines forming a loose reticulum indicative of some swelling. Ordinarily they fail to stain with eosin and methylene blue though they take the blue if the staining is forced.

The cells responsible for the gray or black hue of many virus papillomas, and of a few of those due to tar, are melanoblasts for the most part (Figs. 34, 35), though the epithelium in the lower part of the proliferating layer often contains brown granules. The melanoblasts are frequently black with pigment granules which tend to be larger, coarser, and darker colored in the virus growths; and such cells accumulate much more slowly in the tar tumors, only coloring those which enlarge very gradually. The exceptional tar papillomas which were almost black had become sq in the course of months during which they scarcely changed in size. Gray tar tumors which begin to grow fast soon become pink. The proliferation of the melanoblasts of virus papillomas, on the other hand, frequently keeps pace with their enlargement, and in consequence they remain sooty throughout months of vigorous proliferation (2). The melanoblasts are no essential constituent of the growths, however, but are included fortuitously (16), and they may sooner or later disappear or be outstripped (17). The same holds true in more striking degree of the tar papillomas, and the melanosis is of essentially the same sort.

Any irritation of the skin of gray-brown rabbits may bring on a graying of skin previously pink, and long-continued tarring often results in black freckles (Fig. 1), due to aggregates in the corium of chromatophores stuffed with pigment. Sometimes the source of this can be traced to a distant tar tumor or patch of epidermis containing active melanoblasts or epithelial cells in which pigment is abundant. Where sooty tar or virus tumors have disappeared a dark patch may persist, due to residual melanin in melanoblasts and chromatophores. Such patches were especially frequent in the case of the virus tumors (Fig. 46), and phagocytes dark with pigment were sometimes present in the lymph nodes into which they drained.

The shape of tar papillomas is markedly affected by continued tarring. Many become pedunculated (Figs. 40, 43), the proliferating connective tissue underneath the growth lifting it away from the ear. This happens less frequently with virus papillomas, because their cells multiply faster, are more aggressive, and hence can maintain their original place near the cartilaginous plate. Yet a pedunculation of virus papillomas may occur, even in the absence of any tarring, if they become indolent (Fig. 42) (12), and it often takes place despite rapid enlargement when tarring is done (Fig. 39). The likeness to pedunculated tar tumors may then be complete at low magnifications (Figs. 39 to 43) as well as in the gross. Virus papillomas on the rabbit's side have never become pedunculated in our experience. There the fibrous corium is a thick, tough sheet which does not yield to the weight even of huge growths, while furthermore none of our animals was tarred in this situation.

The virus papillomas of some rabbits are fleshy, whereas those of others, although doing well, are dry almost to the skin (Figs. 2, 3). This is true also of tar papillomas. When growths of both origins coexist on the same ears they frequently vary together in such respects, host influences obviously affecting them in the same way.

Retrogression.—When tarring has not produced enduring effects on the skin most of the growths due to it dry down, scab, and the scab comes away leaving a smooth scar (1). Some drying down and disappearance take place in any case unless the tumors are crowded and macerating. Most of the papillomas which persist become deeply cleft, brittle cauliflowers, or petaloid excrescences with constricted bases, or else horns or narrow cones, dry nearly to the skin. A few remain fleshy, however, and these usually have an onion shape. Some growths reconstitute themselves from beneath after scabbing. These exhibit no peculiarities, nor do such papillomas as may appear later. Generally virus papillomas are much less affected by the discontinuance of tarring, as might be expected from their great vigor; and many continue to grow rapidly, and remain of onion shape or broadly conical. Some, however, assume the cauliflower or petaloid form, or become high horns.

The retrogression of tar and virus papillomas is attended by the same microscopic changes. When it is very slow the protruding, finger-like papillae become fewer and narrower, the layer of living epithelium shallower, the underlying connective tissue denser, and eventually the mass consists almost entirely of keratin (Figs. 42, 43). These changes, largely referable to sclerosis of the supporting tissues, are especially frequent in pedunculated growths.

When retrogression is rapid the growths get lower, owing to an unrepaired loss of keratinized material, their papillae shorten, and instead of broad "elephant's feet" of living epithelium along their base one finds narrow, irregular processes, separated and underlain by a reactive connective tissue containing many macrophages and lymphocytes (Figs. 44, 45),—cells almost absent from the slowly dwindling growths just described, and infrequent in tarred skin returning to normal. The epithelial processes appear as if invasive, and mitosis is still going on in their cells (as in retrogressing mouse carcinomas also (18)); yet the growths are actually smaller and more superficial than before, connections with the sebaceous glands and hair follicles have appeared (Fig. 44), and the underlying cellular accumulation (macrophages, lymphocytes) is of the sort which accompanies the retrogression of tumors generally. At length only a smooth scar is left, covered with somewhat thickened squamous epithelium devoid of distinctive peculiarities.

After tar carcinoids and papillomas are implanted in the subcutaneous tissue or leg muscles of the host they either die by keratinization or round up into small cysts, as already stated. The same holds true of virus papillomas implanted in hosts which develop a resistance to these growths (19). In both cases the living epithelium at the periphery of the cysts ceases to have a neoplastic appearance.

Conditions of Origin.—The real cause of the tar papillomas is something other than tar. The virus on the other hand is directly responsible for the papillomas it produces. The unknown causes of the tar tumors in general, and of the growths due to other "carcinogens," act only because of some change of a peculiar nature that these agents slowly bring about in the tissues; whereas the virus, though rendered unusually effective by this change (1), can act upon epidermis which has been merely scarified or acutely injured in other ways, as by an application of sodium bisulfide (10), or by inflammation due to a subcutaneous abscess. In contrast to the causes for the tar tumors the virus needs little help to be pathogenic, and only non-specific help, while the growths it produces need no help whatever in most instances. Nevertheless one may recall, as bearing on the possible causation of tar tumors, that tarring brings about conditions exceptionally favorable to localization of the virus out of the blood stream, that it enables it to engender growths after introduction into normal skin,—when otherwise this would not happen (10),—and that it exerts a stimulating effect upon virus papillomas which is often pronounced, though not ordinarily decisive for their fate as it so frequently is in the case of tar tumors.

The ears of some rabbits are especially sensitive to tarring; and the more markedly it changes them the more likely are tar tumors to arise. This holds also for the papillomas which result from an intravenous injection of virus, though the conditions making for localization of the virus under such circumstances must be discriminated from those determining the formation of tumors (10). When both ears have been tarred equally, tar tumors appear in approximately the same number on both. So it is also with the papillomas arising after virus has been thrown into the blood stream: the average for both ears is the same unless the circulation to one was interfered with while the virus was circulating (10).

The tumors due to tar are local epithelial phenomena, the outcome of happenings at special points in the hyperplastic epidermis. So also with the virus tumors, though when an immense quantity of virus has suddenly been placed in circulation it may infect the tarred epidermis at so many spots as to give the impression that a generalized neoplastic transformation has occurred. Theoretically one should occur if the virus reached and infected every cell in the basal layer of the stratum germinativum. Actually, however, what appears to be generalized neoplastic change after massive virus infection, is usually due to a secondary coalescence of growths arising at numerous, separate points, as sections taken at early stages have proved.

The increase in size of the papillomas due to virus is due to multiplication of the cells with which it becomes associated primarily, not to contact infection of adjacent elements (12). This holds good also for the tar tumors. Both are frequently multicentric in origin, but the tar tumors only exceptionally arise in hordes. In an unique instance, the ears of a rabbit tarred in the usual way for 110 days were practically covered with tumors, close crowded and becoming confluent. In a few small blocks taken at random 61 papillomas could be discerned, as also 2 carcinoids and 2 frill horns. These findings were not included in Table I, to avoid distortion of the figures. The ears of an animal examined after tarring throughout 13 months showed large expanses of low, diffuse papillomatosis, which might have been interpreted as the result of a generalized change in the epidermis had there not been charts proving it due to a coalescence of growths originally separate and discrete.[1]

After an intravenous injection of virus, hosts of papillomas often arose on the outsides of ears rendered hyperkeratotic by tar transferred from the insides, and on the neck as well, where similar changes had taken place for the same reason.

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[1] Suntzeff, Burns, Moskop, and Loeb, studying the downgrowths of uterine epithelium in mice given an estrogen (*Am. J. Cancer*, 1938, **32**, 256), have noted that they often occur at many points, and sometimes everywhere as if by a generalized transformation. Their conclusion that these findings are scarcely compatible with the working of a virus, would seem unwarranted in view of our observations.

The growths were marked as due to the inoculum by their appearance almost simultaneously after the usual incubation period, by their rapid growth and frequent, deep melanosis, by their cytology, and by the almost complete absence of tumors from such situations in animals merely tarred, unless the tarring had been kept up for many months (15). When this was the case scattered tar papillomas eventually appeared on the neck and outsides of the ears, and they derived from the same structures and took the same forms as the virus papillomas (Figs. 21 to 30). The earlier appearance there and greater frequency of virus-induced growths were obviously referable to the relatively slight epidermal changes required for the action of the virus, to the abundance of it provided, and to the vigor of the growths it caused.

The tumors due to tarring occur at irregular intervals, and new ones may keep on appearing for months or years afterwards, if the skin has been rendered enduringly pathological. Nearly all of the growths due to virus, on the other hand, appear within a few weeks of the intravenous injection unless the skin has undergone such change. Under these circumstances characteristic virus papillomas may continue to appear sporadically for at least 4 months after the inoculation, a period double the longest observed in the case of normal skin. In one experiment a rabbit's ears were acutely inflamed by swabbing them with barium sulfide, virus was injected intravenously, and 120 days later tarring was begun. At this time there were 3 papillomas present, which had appeared shortly after the injection, but later, as tarring went on, a fourth developed, shortly before the 160th day. Like the others it was marked as due to the virus by the retention of a slaty hue despite rapid enlargement, as furthermore by its characteristic cytology.

Course.—Tar papillomas may run widely differing courses though all are markedly responsive to change in the general state of the ears. Some of the earliest to arise and the swiftest to grow may vanish despite continued tarring, and while others nearby are rapidly enlarging. Some, beginning late and growing slowly, continue to grow after tarring is left off though all their neighbors are disappearing. The majority, however, enlarge or retrogress together. So too do the virus papillomas resulting from a direct inoculation of normal skin; for they are mostly multicentric in origin and hence what they do constitutes an average response. But when the virus is distributed on the blood stream,—providing, as this does, an opportunity for its individual entities to infect single cells,—and the state of the cells has been altered by preliminary tarrings, the growths may not only appear at widely differing times and vary much in neoplastic character (10), but often certain of those which appear to be typical virus papillomas may grow vigorously while others wholly resembling them and situated on the same ear are retrogressing. They now differ in incidence and course as do the tar papillomas, though not to nearly so great an extent. This is scarcely surprising in view of the fact that the virus exerts much more compulsion on the cells than does the cause for the tar papillomas, and is far less dependent on contributory circumstances for its continued action, as expressed in behavior of the growths. What the virus does is of predominating importance for the course of neoplastic events, once these are under way, not what attendant or intercurrent circumstances do, as in the case of most tar tumors.

The superior pathogenicity of the virus is usually evident in the behavior, as well as the form, of the papillomas engendered by it: they enlarge more swiftly than most tar tumors and appear relatively unaffected when tarring is discontinued. Nevertheless in some animals they may retrogress all at once, a host of growths which have long flourished every one vanishing, perhaps within a few days, even though tarring is kept up. This behavior is due to the development of a generalized host resistance (20), which is not always comprehensive in its effects when the papillomas are situated on tarred ears, some of them continuing to enlarge in contrast to the majority. And even when the resistance is all-inclusive it is often transient, some growths reappearing after a few weeks in their previous positions, marked by patches of pigment in the corium. Tracings were made on cellophane in a few cases while the growths were dwindling, in order to record their exact situations; and after they had vanished tarring was begun anew. Some reappeared, but one cannot be sure that the tarring was responsible since recurrence can take place without it (20). Recently we have found that renewed tarring may call forth tar tumors where they were before, months after seemingly complete disappearance.

Whether any host resistance develops to tar papillomas is uncertain. The wholesale retrogression often witnessed after tarring has been stopped is plainly due in most cases to involutionary changes in the skin of the ears; and the sensitiveness of the tumors to these latter renders it difficult to tell whether other adverse influences are working upon them. Certainly host resistance, if such there be, is much less comprehensive and effective than that provoked by the virus tumors, which is notably selective, frequently causing complete disappearance of virus papillomas while exerting no visible influence upon the tar tumors associated with them.

Spurious Malignancy.—The progressively increasing disturbance of the skin, when tarring is kept up, acts to further the growth of tar papillomas, to disorder their form and to render them aggressive. Some extend deep, forming numerous small cysts secondarily, while the pattern of others becomes irregular and complex. They may penetrate to the outside of the ear and form secondary masses there, even while retaining their original morphology (Figs. 37 and 38). Not a few of them break up along the base as if becoming carcinomas, though actually they are but carcinoids in most instances; and growths of the latter sort keep on appearing. Yet if tarring is stopped after 2 to 4 months all of the apparently malignant tumors take on the form of benign papillomas, or become cystic or disappear. It is plain that tar tumors are very prone to spurious malignancy.

Virus papillomas are much less sensitive to continued tarring, though under its influence they too may become somewhat disorderly, extend down and form cysts, or exhibit complexities of pattern. Often they become very aggressive and grow through to the outside of the ear (Fig. 36). Yet even when proliferating with prodigious rapidity they do not assume the carcinoid form, although it is seen when the growth is implanted in the interior organs, especially under conditions of local inflammation (12). The virus evidently exercises a stricter formative influence upon the cells with which it is associated than does the cause of most tar papillomas. Yet the formative influence of this unknown cause is notably strict in not a few instances, the growths remaining papillomas, however much tarred, and under circumstances which lead others immediately next to them to be carcinoids (Fig. 24).

So long as tar and virus papillomas continue to be such they retain the cytological features distinctive of them, no matter how complicated or disorderly their pattern; but most of these features only become manifest as differentiation takes place, and they are lost in proportion as the cells fail to differentiate, as when they become carcinoids or carcinomas. Markedly anaplastic growths deriving from tar and virus papillomas cannot be distinguished as of differing origin. The character of the cells in the papillomatous part of the cystic metastasis shown in Fig. 20 identified the growth as a tar tumor, whereas the cells of its invasive portion yielded no information in this regard.

The Development of Carcinomas.—The course of events when tarring was discontinued after a few months proved that all of the apparent malignancy then existing was spurious; but this was not the case when tarring had been kept up for a long time or had been done throughout several periods. In 2 rabbits thus treated genuine cancers arose, as already stated, while in others malignant changes seemed under way. The 3 cancers all derived from papillomas, like the generality of these growths in rabbits (4, 6). In the present comparison we are concerned only with cancers of such derivation.[2] The changes which take place when tar papillomas become malignant have often been pictured (4, 6), and they are morphologically identical with those taking place in virus papillomas which become malignant, the carcinoma cells stemming from the papilloma cells by alterations of greater or less magnitude (21). Furthermore the tar and virus cancers exhibit precisely the same forms, some being "papillomas of the second order," some cystic, while others are frankly malignant papillomas, and yet others are more or less anaplastic squamous cell carcinomas (21). A growth may assume all these forms successively, or a form primarily or secondarily assumed may be long retained, perhaps until death of the animal. The metastases of both the tar and virus tumors may be cystic[3] or solid, exhibit papillomatous features (Fig. 20) (4), or be anaplastic.

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[2] Tar readily evokes squamous cell carcinomas in cottontail rabbits, as we have found, and some of the cancers do not derive from papillomas but have the carcinomatous form from the beginning. Yet the possibility cannot be excluded that they originated from carcinoids, themselves a manifestation of the spurious malignancy of papillomas. For carcinoids are frequently elicited in cottontails.

[3] The statement of a previous paper (*J. Exp. Med.*, 1936, **64**, 401) that the metastases of tar cancers are never cystic or papillomatous was based on information since found to be incomplete, and it is negated by the instance of Fig. 20.

The tar cancers derive mostly from those papillomas which are broad-based (4), that is to say from those which are most vigorous; and it is from the most actively growing of virus papillomas that cancers arise oftenest. Continued stimulation of tar papillomas by further tarring hastens the occurrence of malignancy; and it is hastened in virus papillomas by a variety of stimulating procedures (21). Yet these are not essential in either instance. The vigor of virus papillomas as a rule greatly exceeds that of those due to tar; and, as might be expected, they become malignant much more often, and generally after a much shorter period, while the carcinomatous change is frequently multiple and cancers sometimes appear almost simultaneously at many spots in a single growth (21). Only an occasional tar papilloma ever becomes malignant.

Tarring so stimulates neoplastic proliferation in general that it may conceivably bring into the open some cancers which would not otherwise assert themselves, and which cannot progress after it is stopped. Yamagiwa and Ichikawa elicited a tar tumor with the morphology of a carcinoma, which metastasized yet retrogressed after 630 days (4); and amongst the numerous tar tumors of cottontail rabbits that we have recently evoked squamous cell carcinomas requiring aid have been frequent, growths which enlarged so long as tarring was kept up, not infrequently destroying a great part of the ear, yet which dwindled, though retaining the morphology of cancers, after it was left off, and eventually disappeared. In domestic rabbits growths of this sort are evidently rare, though the case just cited shows that they occur. Two large ulcerating cancers which had derived "spontaneously" from virus papillomas in our domestic rabbits, and were squamous cell carcinomas on biopsy underwent secondary, retrogressive changes. One had existed for months as a large, fungoid, malignant papilloma, distinctively different from the rest of the papillomatous mass amidst which it originated. Eventually it grew smaller and was almost replaced by the surrounding papillomatous tissue. The other cancer, an anaplastic, metastasizing squamous cell carcinoma, was at one time a bulky, ulcerated growth, yet dwindled in the end to a mere puckered induration over which the skin had healed. The microscope disclosed, however, that nests of living carcinoma cells still existed amidst a sclerotic connective tissue.

DISCUSSION

The benign tar tumors here described and classified were sharp cut pathological entities. They were not local exaggerations of the epidermal hyperplasia due to tarring, nor were they random neoplastic manifestations. One did not find papillomas grading into frill horns or *vice versa.* Whatever the nature of the cause for the frill horns, it expressed itself with an exquisite particularity, making the cells do precisely thus and so, with result in highly distinctive growths. The cause for the tar papillomas, though also acting upon the cells of the stratum germinativum, produced tumors of wholly different sort, which usually expressed themselves in a characteristic, benign form, yet were often so responsive to external conditions as to assume instead a carcinomatous aspect without undergoing real cancerous change.

A comprehensive mapping of all the neoplastic potentialities of several animal species would greatly aid thought on tumor causation. But even in man only those potentialities are known today which have become actualities as result of the "carcinogenic" accidents of life. Observation has been largely haphazard in the case of other creatures, yet it already points to remarkable species differences. Tarring the skin of dogs, for example, results in malignant melanomas, apparently with some regularity (22), growths seldom if ever evoked in the rabbit or mouse. Tarring the skin of cottontails never results in frill horns, though frequently evoking papillomas, carcinoids, and cancers, as our extensive observations have shown. Can it be that the epidermis of different species of animals has different inherent potentialities for tumor formation? Or are these potentialities not inherent but due to agents of extraneous origin? Such questions can only slowly be answered.

The study here presented was made with hybrid rabbits of one sort (agouti) and with a single tar; but recently it has been extended to another breed (Dutch show rabbits). In these tar also evoked frill horns, papillomas, and carcinoids, with no other benign tumors, and in approximately the same relative number as in agouti rabbits. Furthermore a different tar has been applied to agouti rabbits and again the same tumors have been called forth, this time in an epidermis stimulated to much greater general change. The finding might have been expected in view of the evidence that tars owe their carcinogenic action to a common constituent, 1:2 benzpyrene. The pictures in the literature show that the domestic rabbits of England, America, Italy, Scandinavia, and Japan all yield on tarring papillomas and carcinoids resembling in general those with which we have dealt. But the differing fixatives and stains used on the specimens prevent any decision on whether the papillomas had the morphology of those of our animals. Some appear unlike them in details. Orr (23) reports that the growths evoked in mice by six carcinogenic hydrocarbons resemble those due to tar. It would be worth while to know the neoplastic effects of a single carcinogen upon rabbits of identical sort, bred in widely separate parts of the world, living under different conditions and fed different foods. (Our rabbits, all procured in New York, were housed and fed alike.) But in any typing of the growths thus evoked it would be necessary to discriminate their essential features from those due to intercurrent influences. This has not been done in the past, but instead all of the features of the tumors have been taken to be the expression of innate, individual peculiarities, and in consequence they have been deemed much more various than they actually are.

Because of the collateral influences that tar exerts it is far more effective in producing tumors in rabbits than any of the pure carcinogenic substances (10). It evokes growths much sooner than does benzpyrene even when this is applied in relatively large quantity, and it brings about local tissue changes which enable the tumors to become established. Directly or indirectly it urges them on and produces secondary changes in them. Because of this last influence tar tumors are best studied when young. Had we not examined them then, the narrow restrictions in their types might have been overlooked and the reasons for their later complexities have been missed.

The main facts emerging from the comparison of the benign tar and virus tumors are set forth in Table II, together with certain inferences which seem unavoidable. Our study was carried out on domestic rabbits, instead of cottontails, the natural hosts of the virus, to exclude all possibility that some of the growths evoked by tar might be due to a strain of virus lying latent and of such slight pathogenicity as to require the aid which tarring gives. For it is known that the papilloma virus may lie latent after introduction into normal skin and give rise to papillomas when tarring is done (10). The virus is wholly foreign to domestic rabbits and it cannot ordinarily be got again from the growths engendered in this species. Nevertheless the tarred rabbits were isolated to rule out contact infection entirely.

Serological tests indicate that the cause for the tar papillomas is not antigenically related to the Shope virus (24), and the distinctive cytological changes that it produces accord with this finding. To account for the general likeness to the virus tumors, one might assume that anything which stimulates proliferation of the cells of the stratum germinativum will give rise automatically to papillomatous growth, if the proliferation is superficial. One may recall in this relation the likeness of silicosis tubercles to those due to bacillus

TABLE II*a*

Comparison of the Benign Tar and Virus Tumors Deriving from the Epidermis of Domestic Rabbits

<table>
<tr><th><i>The Tar Tumors</i></th><th><i>The Virus Tumors</i></th></tr>
<tr><td>Tarring evokes the growths</td><td>Tarring results in a localization of circulating virus: it enables latent virus to produce growths</td></tr>
<tr><td>Peculiar, chronic, "carcinogenic" tissue disturbances bring the unknown causes into action</td><td>Various non-specific, acute or chronic tissue disturbances render the virus effective</td></tr>
<tr><td>The growths appear on the tarred skin at irregular intervals</td><td>After a single intravenous injection of virus the growths all appear within a few weeks unless the skin has been often tarred, when they may appear irregularly</td></tr>
<tr><td colspan="2">Number and time of appearance vary from host to host, but incidence is similar on both ears</td></tr>
<tr><td colspan="2">Origin punctate or focal, but often multicentric</td></tr>
<tr><td colspan="2">Enlargement takes place by intrinsic cell proliferation (<i>aus sich heraus</i>)</td></tr>
<tr><td>The tumors are largely dependent for persistence and growth on further tarring or on chronic changes already induced in the supporting tissues</td><td>The growths can progress without help of tarring or of chronic tissue changes, though aided thereby</td></tr>
<tr><td>Individual rates of proliferation vary widely; implantation elsewhere in the host is unsuccessful</td><td>Proliferation usually very rapid; implantation successful</td></tr>
<tr><td>Unfavorable local conditions frequently bring about general retrogression</td><td>Local conditions seldom cause general retrogression</td></tr>
<tr><td>Growths of two distinct types,—(1) papillomas subject to spurious malignancy = carcinoids, (2) frill horns</td><td>Growths are of a single sharply defined type,—papillomas</td></tr>
<tr><td colspan="2">Inclusion bodies absent: no distinctive changes in the supporting tissue</td></tr>
<tr><td colspan="2">Morphology indicates that they are due to specific causes, each with characteristic effects</td></tr>
<tr><td>Causes have not been recovered from the growths</td><td>Usually the virus cannot be recovered from the growths induced with it</td></tr>
<tr><td><i>The unknown causes require special cell conditions for their action, and exert little compulsion</i></td><td><i>The virus cause is very compelling and does not require special aid</i></td></tr>
</table>

TABLE II*b*

<table>
<tr><th>The Tar Papillomas</th><th>The Virus Papillomas</th></tr>
<tr><td colspan="2">Both are sharply defined pathological entities</td></tr>
<tr><td>Arise mostly from the deeper portion of the hair follicles</td><td>Arise mostly from the deeper portion of the hair follicles when the virus localizes out of the blood stream</td></tr>
<tr><td colspan="2">Arise from the stratum germinativum</td></tr>
<tr><td colspan="2">Origin frequently multicentric</td></tr>
<tr><td colspan="2">Tumors enlarge in same way, assume same gross forms; virus papillomas usually more fleshy because growth more rapid</td></tr>
<tr><td colspan="2">Superficial resemblance close but cytology distinctive</td></tr>
<tr><td>Occasional melanosis, and melanoblasts mildly stimulated. Pigmentation lost during rapid growth</td><td>Melanosis frequent, and melanoblasts greatly stimulated. Pigmentation often retained during rapid growth</td></tr>
<tr><td colspan="2">Melanosis is of similar character</td></tr>
<tr><td colspan="2">Histological phenomena similar during retrogression</td></tr>
<tr><td colspan="2">Peculiarities of individual host influence gross form in same ways</td></tr>
<tr><td>Aspect and course markedly influenced by local conditions</td><td>Aspect and course moderately influenced by local conditions</td></tr>
<tr><td colspan="2">Continued tarring renders growths aggressive, complicates morphology</td></tr>
<tr><td>Growths highly responsive to tarring. Spurious malignancy (formation of carcinoids) is a frequent result</td><td>Growths less responsive to tarring. It fails to induce spurious malignancy but other influences do this frequently</td></tr>
<tr><td>Retrogression usual unless chronic changes have been induced in the supporting tissues</td><td>Retrogression not infrequent; chronic tissue changes aid persistence</td></tr>
<tr><td>General retrogression frequent owing to local conditions; whether an induced resistance ever brings it about is uncertain</td><td>Local conditions have relatively slight effect, but general retrogression is frequent as result of induced resistance</td></tr>
<tr><td colspan="2">Growths may reappear after vanishing</td></tr>
<tr><td>Pathogenicity of cause, as expressed in tumor behavior, varies much from growth to growth</td><td>Pathogenicity of virus, as expressed in the multiple growths of a single infection, varies little</td></tr>
<tr><td>Occasional carcinomatous change after many months</td><td>Carcinomatous change frequent after a few months</td></tr>
<tr><td colspan="2">Intercurrent stimulation hastens the malignant change</td></tr>
<tr><td colspan="2">The cancers arise from the papilloma cells by similar morphological alterations and exhibit the same general characters</td></tr>
<tr><td>The cause of the papillomas stimulates the cells mildly and exerts a formative influence upon them which is often overborne. It has a moderate carcinogenic effect</td><td>The virus stimulates the papillomas greatly and exerts a strict formative influence upon them. It has a pronounced carcinogenic effect</td></tr>
<tr><td colspan="2">The virus seems not to be related antigenically to the tar tumor cause yet the differences in the neoplastic phenomena they induce are merely quantitative</td></tr>
</table>

tuberculosis. But tar and virus papillomas exhibit their characters though growing beneath the skin (Figs. 29, 30, 36, 37), while furthermore the frill horns, though submitted to similar mechanical influences and deriving from the stratum germinativum of hair follicles (Fig. 31), like the growths just mentioned, have a wholly dissimilar morphology (Figs. 8, 9).

Some of the tabulated differences in the tar papillomas and those due to virus are obviously consequent upon the great pathogenic activity of the latter, to its introduction into the test rabbits upon a single occasion, and to its almost standardized effects under ordinary circumstances. There is every reason to suppose that if we had mixed several virus materials of differing pathogenicity and injected the mixture several times, dribbling it into the animal, so to speak, the individual differences in incidence and behavior of the tar papillomas would have been duplicated.

The virus is a much more exigent formative influence than the unknown cause of the tar papillomas, the growths it produces retaining their morphology under conditions which would cause many tar papillomas to become carcinoids; but this difference would appear to be merely quantitative, since virus papillomas also become carcinoids on special occasion. In both instances the apparent malignancy means only that epithelial cells subjected to extraneous stimulation can simulate malignant cells, a fact already proven for normal epithelium by the invasive downgrowth with anaplasia which occurs after intradermal injection of Scharlach R or Sudan III in olive oil.

Though spurious malignancy is much more frequent in tar papillomas than in those due to virus, they are far less likely to undergo real malignant change, and the change generally takes place much later. The frequency with which cancer develops out of virus growths varies directly with how hard their cells are driven by the virus, as manifested by their rate of enlargement (25). The more rapid the proliferation, and the more the cells are played upon by extraneous stimuli (*e.g.* incision, Scharlach R injection, inflammation due to bacterial infection or vaccinial necrosis), the sooner and oftener does malignancy occur. The unknown cause of the tar papillomas exerts only a mild and conditional compulsion, as demonstrated by the behavior of the growths. This being so, one might expect that cancer would arise late and infrequently from tar papillomas, as is generally the case. Yet to all appearance malignancy is the outcome of precisely the same train of events as in virus papillomas that are becoming malignant; and the resulting carcinomas are of the same kind and exhibit the same limited diversity. In a previous paper (21) we have discussed certain human cancers which arise from papillomas by changes resembling those which occur in rabbit papillomas, and have cited furthermore a virus of man which causes papillomatous proliferation out of which squamous celled carcinomatosis occasionally arises, namely the virus responsible for condyloma acuminatum (26). Like the unknown cause of the tar papillomas this virus fails to produce growths unless aided. Uncleanliness, local bacterial infection, tissue maceration, act to render it effective and to maintain the resulting condylomas, just as tar acts to evoke and maintain the tar tumors; and the cancers arise, as in their case, out of growths subjected to long-continued disturbance (27).[4]

SUMMARY

Tarring the ears of rabbits of one sort with a single kind of tar evoked epidermal tumors of a few sharply defined types, namely ordinary papillomas, carcinoids, carcinomas, and "frill horns." These last, relatively infrequent, are now recognized for the first time. The carcinoids have proved to be the expression of a spurious malignancy of papillomas, resulting from intercurrent influences, and they were wholly dependent upon these for their threatening aspect and behavior. Chief amongst such influences was continued tarring. It had the effect of establishing the papillomas, stimulated their proliferation, complicated their morphology, and rendered some of them disorderly, aggressive, and anaplastic. It brought all of the tissues of the ears into an excitable state, and often this state endured long after the skin had apparently returned to normal.

The characters of the papilloma-carcinoids and of the frill horns were so different and distinctive as to imply the action of differing, specific causes.

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[4] The treatment of condyloma acuminatum has improved so greatly of late that secondary carcinomatosis has become exceedingly rare; but the conditions of its origin and development are amply documented by the numerous, well illustrated papers on the theme (27).

The papillomas were very like those induced with the Shope virus, and hence a point-to-point comparison was made of their manifestations, including the derivation of carcinomas from them. This comparison demonstrated that the unknown cause of the tar papillomas provoked neoplastic phenomena which were identical in all essential respects with those due to the virus.

To suppose, for experimental purposes, that the papillomas which tarring elicits are caused by a virus rendered pathogenic by this procedure, is to demand least of the unknown. Yet it does not follow that they must be due to a virus.

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EXPLANATION OF PLATES

All of the sections were stained with methylene blue and eosin.

V, T = virus and tar papillomas, respectively.

PLATE 20

FIG. 1. Tar papillomas. The inside of the ear had been tarred throughout several periods of some weeks each, with intervals between for recovery. Photographed on the 591st day, long after the last tarring. The skin between the growths is still somewhat scurfy in places. The tumors are far less fleshy than if tarring had been kept up, and hence do not resemble so closely the virus growths of Figs. 2 and 3. All those along the edge of the ear are papillomas, some medium gray (G), two almost black (G′). Several freckle-like patches of sooty, intracutaneous melanosis can also be seen. Near the middle of the ear is a small, recurved frill horn (H), and at one spot close to its edge is an aggregate of small subepidermal cysts (CY) resulting from the retrogression of a carcinoid. × ½.

FIGS. 2 and 3. For comparison with Fig. 1. Growths produced by directly infiltrating normal ears with papilloma virus by way of a marginal vein, and tarring them three times later, beginning after a week, in order to render the virus effective. (The uninfiltrated ears were also tarred: no growths ever appeared on them.) The papillomas of Fig. 2 were dry, cindery, and nearly all dark gray and slow growing, whereas those of Fig. 3, resulting from the same inoculum, were vigorous and fleshy, and about half of them were pigmented (G, G′). The dry tops of some of the others appear dark. Host influences have a great effect on the form of the growths. × ½.

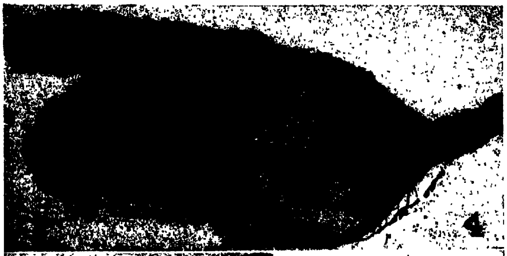
The marginal situation of nearly all of the papillomas of Figs. 1, 2, and 3 is unusual. The specimens were selected for ease in photography.

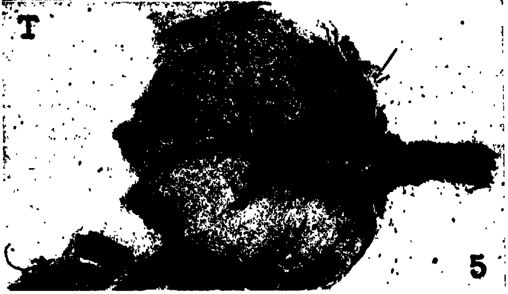
FIG. 4. The melanotic virus papilloma (G′) of Fig. 3,—for comparison with the melanotic tar papilloma of Fig. 5. The edge of the ear happens not to be incorporated in the growth. × 6½.

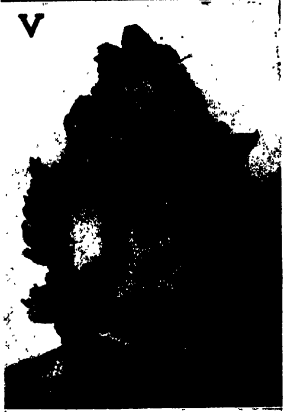
FIG. 5. Cross section of the melanotic tar papilloma, G′ arrow, of Fig. 1. Its outer keratinized portion has been cut away. It extends around the edge of the ear on both aspects, with result that this is incorporated as a spurious raphe. × 6½.

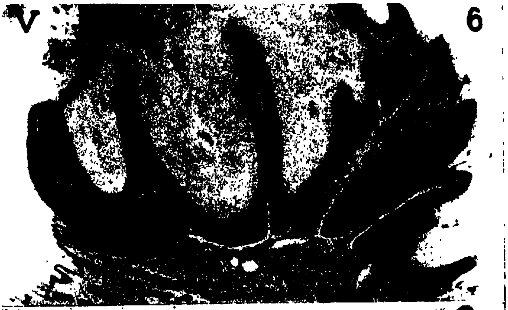
FIGS. 6 and 7. Sections of a virus papilloma and a tar papilloma, respectively (V, T). Most of their dry keratinized portions have been trimmed away. The virus had been directly inoculated into the untarred skin of the side. × 13.

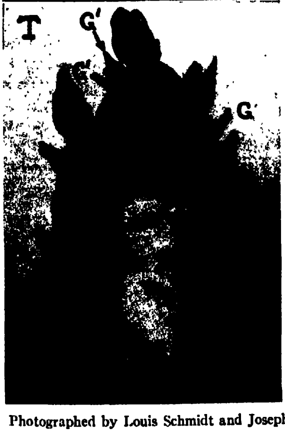
FIGS. 8 and 9. Frill horns, showing the characteristic narrow shape and transverse striation of the dry horns, and the fleshy collar about their bases. The pictures were taken long after tarring had been stopped. × 2½.

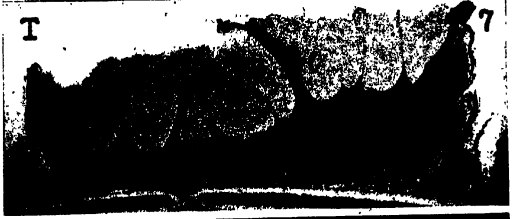


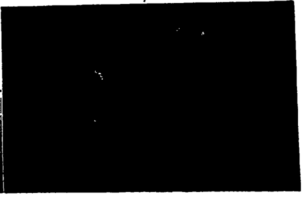












Photographed by Louis Schmidt and Joseph B. Haulenbeek

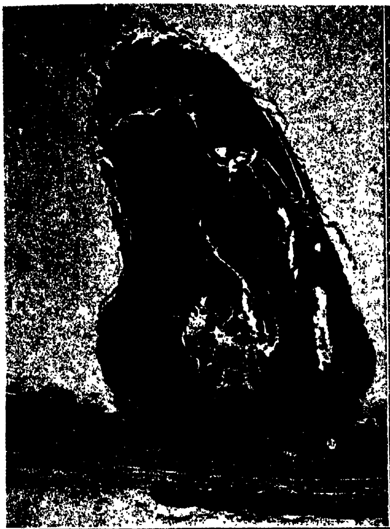
(Rous and Kidd: Comparison of virus-induced tumors with tar tumors)

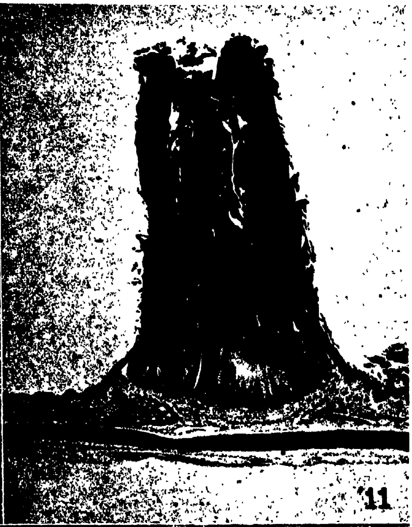
PLATE 21

FIGS. 10 and 11. Small frill horns. The sharply outlined, uninvasive basal frill of living epithelium and the compact, close-textured, keratinized material are alike typical. For convenience in sectioning, the dry horns have been broken off near the base. × 14 and × 10, respectively.

FIG. 12. Part of the base of a frill horn, to show the character of the cells, the absence of a stratum granulosum, and the abrupt keratinization with transient nuclear pycnosis. The scattered, irregular granules stippling the keratinized layer in some places, especially at the right of the photograph, have come from the breakdown of in-wandered polymorphonuclear leucocytes. More macrophages lie beneath the growth than is ordinarily the case. × 190.

FIG. 13. A typical anaplastic carcinoid, selected as illustrating the fact that such tumors are not necessarily preceded by papillomatous proliferation but may extend directly down from the surface epithelium. × 14.





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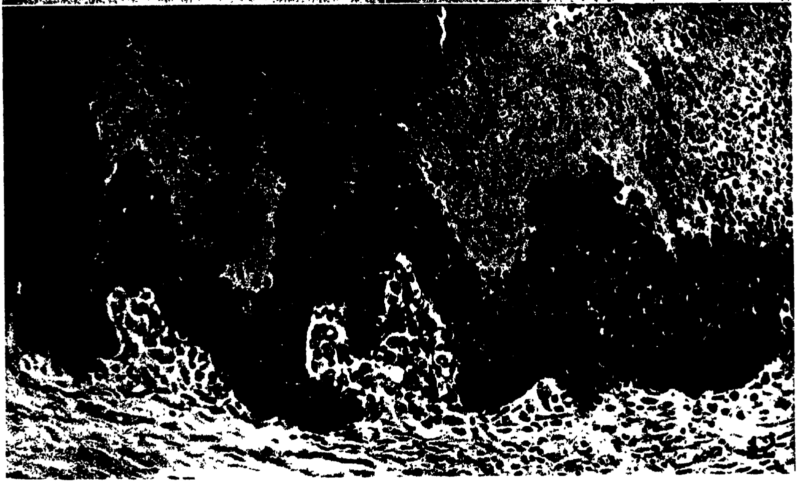

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Photographed by Louis Schmidt and Joseph B. Haulenbeek

(Rous and Kidd: Comparison of virus-induced tumors with tar tumors)

PLATE 22

FIG. 14. A carcinoid of more organized type. × 18.

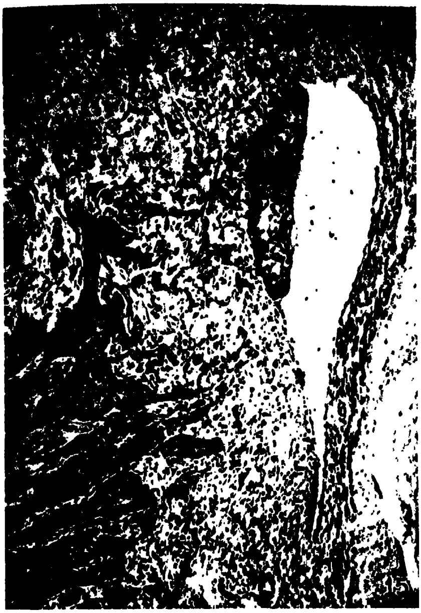
FIG. 15. Invasion of a large lymphatic by an anaplastic carcinoid which appeared after only one month of tarring and was then excised. The reactive connective tissue appears mucoid, as in the case of many squamous cell carcinomas. × 80.

FIGS. 16 and 17. To illustrate the change of a carcinoid to a papilloma after the discontinuance of tarring. Fig. 16 shows a section across a biopsy specimen punched from a large carcinoid 10 days after tarring was stopped. The ear had dried down, and most of the growths, including the carcinoid, had begun to dwindle. It was already losing its anaplastic state, but deep in the reactive connective tissue beneath it were scattered, persisting groups of epithelial cells, not visible in the photograph. Fig. 17 shows the same tumor 28 days later, at the edge of the previous punch hole. Though it has extended around the edge to the outside of the ear, it is now an orderly keratinizing papilloma. The connective tissue beneath it has become sclerotic. × 21.

FIG. 18. Part of an early carcinoid. The rabbit had been tarred only 28 days yet the growth, an ulcerated dome, was already 8 mm. across. Half of it was punched out, as shown. It had invaded a large lymphatic (arrow). At this time there were 14 other growths on the ears, all subepidermal mounds or domes up to 9 mm. in diameter, and two of medium size, as yet unulcerated, were taken *in toto*. They showed carcinoids deriving from an intact epithelium. The ears were now stripped of tar for good. 12 days later only 4 growths remained, 3 as dry scabs, while the fourth, the other half of the carcinoid pictured, was reduced to a mere subepidermal thickening. It was punched out (see Fig. 19). After 2 weeks more all of the tumors had completely disappeared. × 14.

FIG. 19. The rest of the carcinoid of Fig. 18, as it appeared 12 days later. It now consists merely of keratinized cysts lined with stratified squamous epithelium devoid of any obvious neoplastic character. The end of the cartilage at the left marks the edge of the previous biopsy wound, but the scab over the healing tissue here has been torn away, together with a little of it. × 14.

FIG. 20. Section through the wall of a cystic metastasis from a tar carcinoma (see text). The growth was situated in an auricular lymph node. The living epithelium lining the cyst is papillomatous and keratinizing, but further away is anaplastic and notably invasive. × 44.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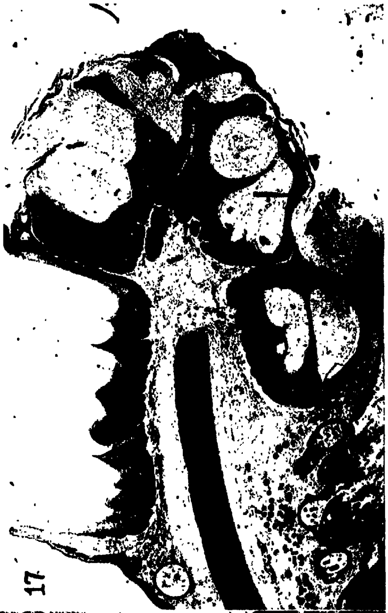
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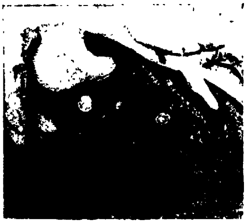

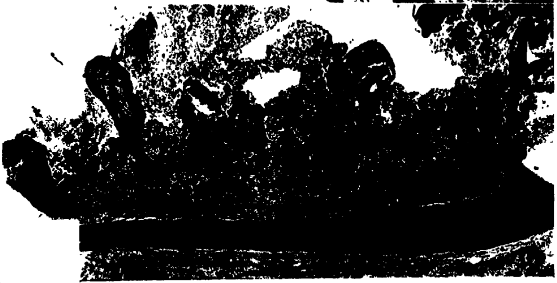

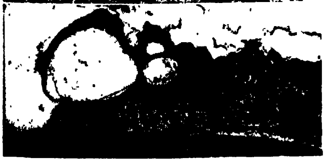

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(Rous and Kidd: Comparison of virus-induced tumors with tar tumors)

PLATE 23

FIGS. 21 and 22. Early stages of papillomatosis due to virus and tar, respectively, — to show that the growths began in the deeper portion of the hair follicles in both instances.

Fig. 21 is from an animal with ears prepared by tarring, which died 22 days after an intravenous virus injection. Massive infection with the virus took place, as shown by a sudden brawny swelling of the ears at about the 18th day (15), followed immediately by the appearance of growths. Autopsy disclosed that they were innumerable, some creamy but many dark. The section is from the outer side of the ear, which had been devoid of tar warts.

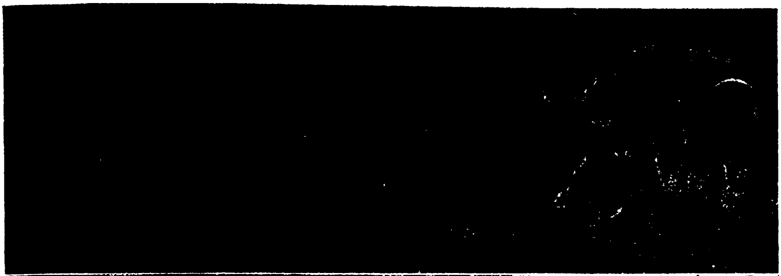
Fig. 22 is from an uninoculated animal which died after it had been tarred for 10 months, intermittently toward the end. At death it had numerous large, pedunculated papillomas on the tarred inner surface and a few small creamy-gray growths on the outside. Some that were just beginning at the latter situation are pictured. × 14.

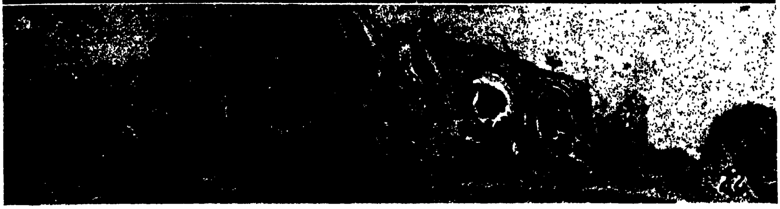
FIGS. 23 and 24. Virus and tar papillomatosis at a slightly later stage. The resemblance is absolute at the magnification shown. A tar carcinoid (C) is included in Fig. 24. × 10.

To obtain Fig. 23, a normal rabbit ear was directly infiltrated with virus and tarred a few times later, as in the case of the ears furnishing Figs. 2 and 3. The specimen shown was punched from the ear 25 days after the infiltration, when innumerable pink or gray growths were appearing on both sides of the organ.

Fig. 24 came from the inside of an uninoculated ear tarred during a period of 80 days. Numerous growths had arisen, both papillomas and carcinoids.

FIGS. 25 and 26. A virus and a tar papilloma, situated on the outside of the ears, and just beginning to erupt. The virus papilloma was the result of a punctate inoculation into normal epidermis. The growth of Fig. 26 came from an animal tarred intermittently for 17 months. × 14.





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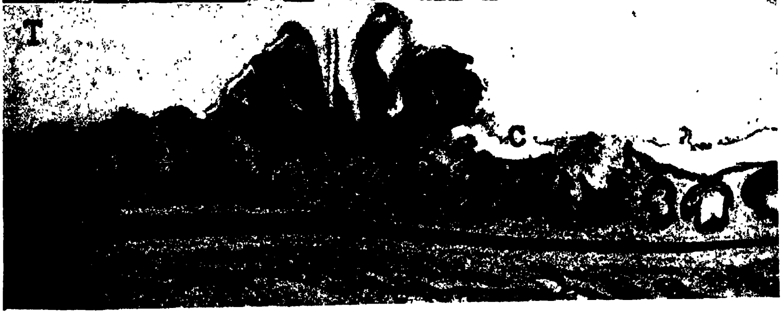

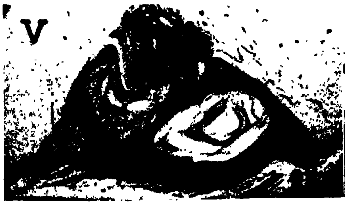

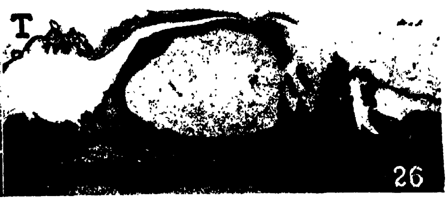

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Photographed by Louis Schmidt and Joseph B. Haulenbeek

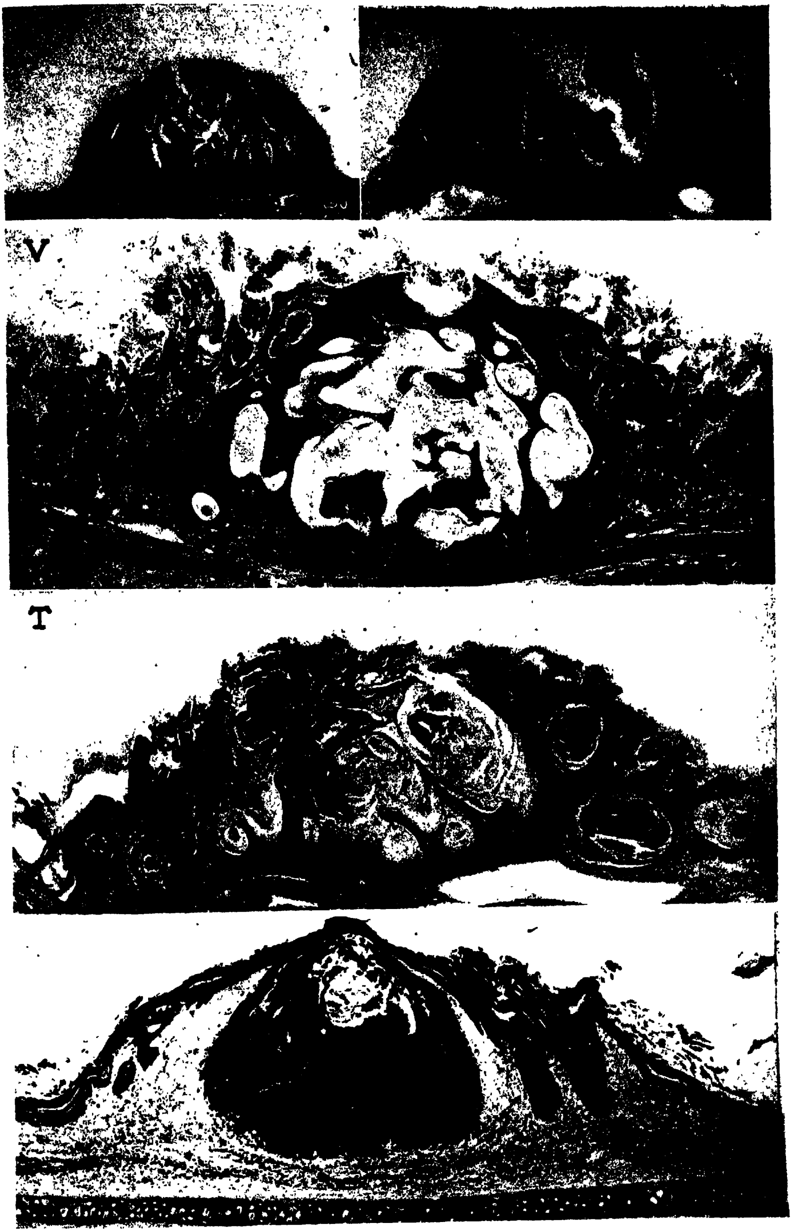
(Rous and Kidd: Comparison of virus-induced tumors with tar tumors)

PLATE 24

FIGS. 27 and 28. Further examples of erupting tar and virus papillomas. The growth of Fig. 27 was due to a punctate inoculation of virus. The tumor elicited by tar (Fig. 28) was from the same rabbit as Figs. 22 and 30. × 6½.

FIGS. 29 and 30. Somewhat larger virus and tar papillomas on the outside of the ear. The papillomatous pattern has become more complicated. The growth due to the virus resulted from direct inoculation, with tarring for 41 days thereafter. Fig. 30, of a tar papilloma, is from the rabbit, tarred for 10 months, that provided Figs. 22 and 28. × 14.

FIG. 31. Early stage of a frill horn; it is just erupting. The dense, strongly eosinophilic, keratinized material contrasts greatly with that formed by the virus and tar papillomas of Figs. 21 to 30, which stains almost not at all. The unstained whorls of keratin at the apex of the growth are the original contents of the distended hair follicle from which it arose. × 30.



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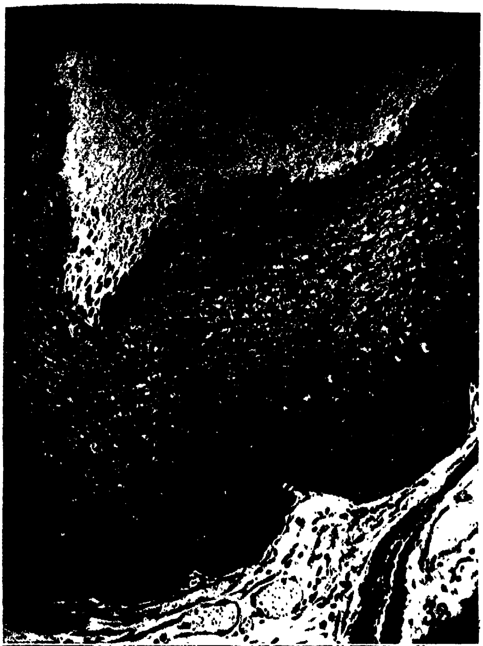
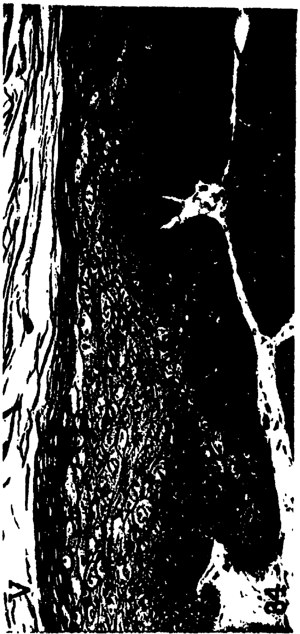
(Rous and Kidd: Comparison of virus-induced tumors with tar tumors)

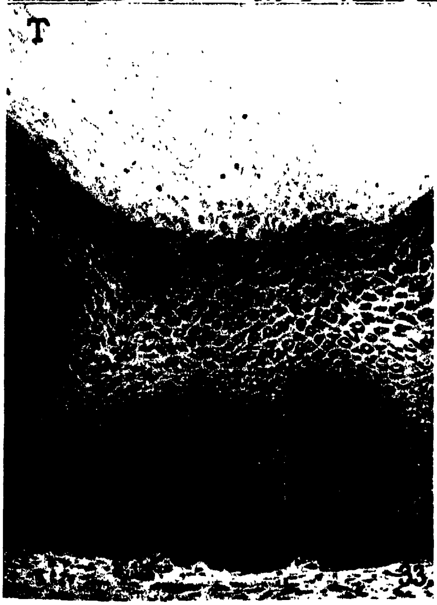
Plate 25

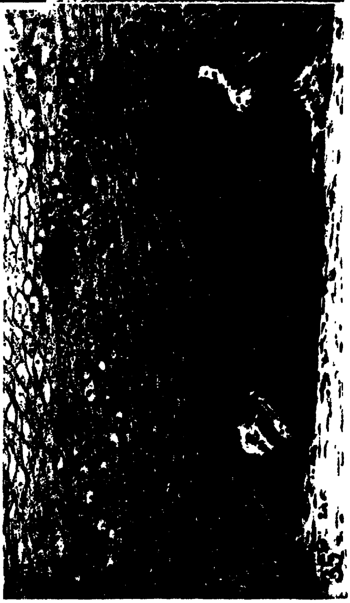
Figs. 32 and 33. The living epithelial layers of a non-pigmented virus and tar papilloma, respectively,—to show the likenesses and differences described in the text. × 177.

Figs. 34 and 35. The living layers of markedly melanotic virus and tar papillomas. The pigment-containing cells are morphologically alike in the two instances and occupy the same situations. × 168.









Photographed by Louis Schmidt and Joseph B. Haulenbeek

(Rous and Kidd: Comparison of virus-induced tumors with tar tumors)

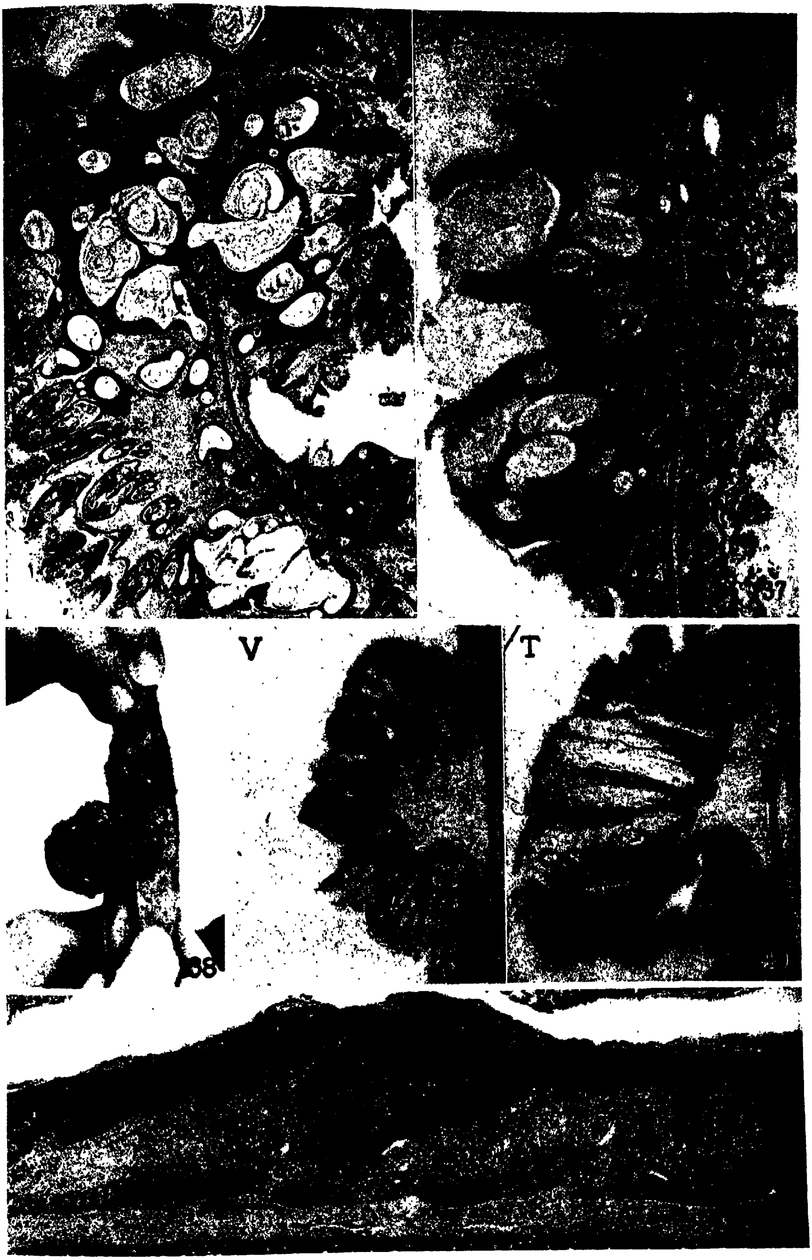
PLATE 26

FIGS. 36 and 37. Virus and tar papillomas which have extended through lacunae in the aural cartilage as result of the stimulus of continued tarring. The virus growth of Fig. 36 was the result of a tattoo inoculation into the inside of a normal ear 73 days previously, with tarring twice weekly thereafter. The growth became fungoid and foul, and extended through to the outside at several situations, of which two are seen here Fig. 36, × 9. Fig. 37, × 13.

FIG. 38. Extension of a tar papilloma to the outside of the ear. A punch biopsy disclosing the character of the growth was done early, and the hole that was left healed completely. The tumor became the large fleshy sphere that is pictured, and while doing so extended to the outside of the ear through the healed wound, with result in a fleshy "onion" there. Both growths consisted of connective tissue for the most part, covered with characteristic papillomatous epithelium, and with embedded islands of the latter. × ½.

FIGS. 39 and 40. Newly pedunculated virus and tar papillomas. The pedunculation was due to continued tarring. The virus tumor had been produced by a tattoo inoculation of a normal ear. × 6.

FIG. 41. Final stage in the retrogression of a melanotic papilloma due to localization of circulating virus in an ear long tarred (15). The growth is almost gone but its place is marked by much intracellular pigment. The skin is everywhere pathological, its epithelium thickened, and the connective tissue unusually cellular. Scattered lymphocytes are present where the papilloma was once situated. × 46.



Photographed by Louis Schmidt and Joseph B. Haulenbeek

(Rous and Kidd: Comparison of virus-induced tumors with tar tumors)

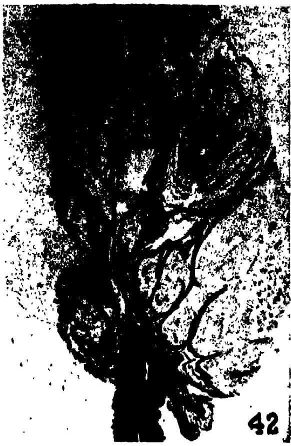
PLATE 27

FIGS. 42 and 43. Old, indolent, pedunculated virus and tar papillomas. The growth due to virus resulted from direct inoculation of a normal ear, and no later tarring was done. It had long since been stopped in the case of the tar tumor. The connective tissue cores of both growths are sclerotic, and masses of keratinized material fill the wide spaces between the infrequent papillomatous fingers. × 3½ and × 4, respectively.

FIGS. 44 and 45. To illustrate the retrogression of virus and tar papillomas. In both cases the living epithelium along the base of the growths has the form of narrow tongues into which lymphocytes have entered here and there; and such elements are fairly abundant in the underlying sclerosed connective tissue, together with macrophages. Connections can be seen between the hair follicles and the epithelium of the virus papilloma. Such connections often reappear when tar tumors retrogress (Ichikawa and Baum, *Bull. Assn. franç. étude cancer*, 1924, **13**, 257), though none is to be seen in the present instance. × 41 and × 48, respectively.

FIG. 46. "Ink spot" pigmentation where numerous melanotic virus papillomas, up to 1 cm. in diameter, had recently disappeared. The spots persisted, and later the papillomas appeared again at several of the situations they marked. × ½.

FIG. 47. Edema on the outer side of the ear opposite a tar carcinoid. There was no extension of the growth through the cartilage. × 6.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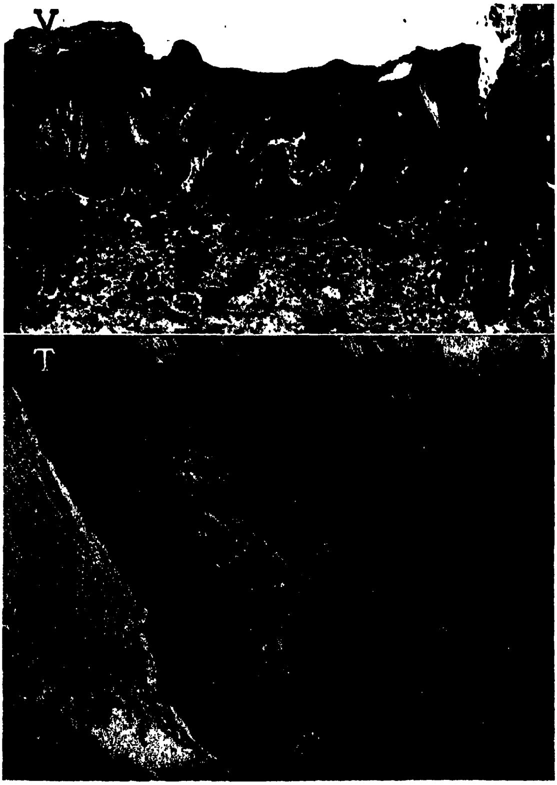

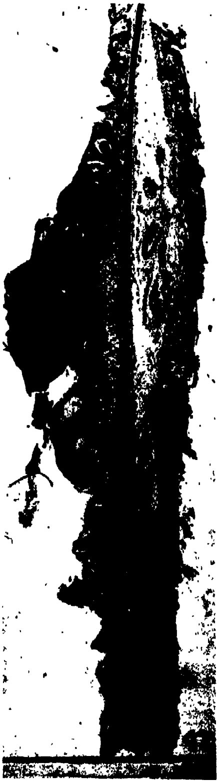

Photographed by Louis Schmidt and Joseph B. Haulenbeek

(Rous and Kidd: Comparison of virus-induced tumors with tar tumors)

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RESISTANCE TO TUBERCULOSIS

I. Factors Associated with the Bacteria

By KENNETH C. SMITHBURN

(From the Laboratories of The Rockefeller Institute for Medical Research)

Until such time as investigation shall uncover highly efficient methods for the specific prevention of tuberculosis, there will be need of an understanding of all the forces which have a part in influencing the course of the disease, either in man or in animals. This need is particularly apparent to workers in experimental tuberculosis, as the ordinarily slow course of the disease makes investigations expensive and progress slow. The prolonged course of the disease may also allow intercurrent factors to play an important rôle, adding to the difficulty of interpretation of results. The need for more precise methods in investigation of tuberculosis was emphasized by Berg who proposed a statistical interpretation of data (1) and, reliant thereon, a standard method for testing vaccines (2). Such procedures would, no doubt, be a step in advance but do not take into account certain factors associated with the mycobacteria, and others associated with the host, which may influence the course of the disease. Toward the end of elucidating some of the latter factors, the experiments reported in this and the succeeding paper were carried out.

The most obvious factors associated with the bacteria which might be expected to influence resistance are virulence of the organisms, the number of them gaining access to the body, and the route by which they enter the body and, consequent thereon, the location of the lesions which they produce. For instance, a diffuse, generalized tuberculous meningitis is associated with early death, whereas an equal mass of tuberculous lesions in the lung or spleen would not cause death except by extension of the process, which would require more time. Medlar and Sasano (3), Winn and Petroff (4), and Steeken, Oatway and Petroff (5) have demonstrated differences in the lesions produced by attenuated and by virulent mycobacteria and we have confirmed and extended these observations (6, 7, 8). As result of these studies, it is now clear that attenuated mycobacteria, when introduced into a susceptible host, induce circumscribed "hard" tubercles which may regress, or which progress slowly, causing death only after a prolonged period (7); but virulent organisms introduced into a susceptible host produce complex lesions which advance rapidly, are markedly prone to necrosis and caseation, and lead to the early death of the host (6, 7).

The intracerebral route of inoculation was used many years ago by Manwaring (9) and more recently and extensively by Feldman (10, 11) and Boquet and Broca (12) in their studies on tuberculosis. We (6, 13) have shown that this method is most useful in quantitative studies on tuberculosis. In the present investigations we have employed the technique previously described (13) in studies on host and parasite factors as they are related to resistance to the disease.

MATERIALS AND METHODS

Animals: The rabbits used in these experiments were, for the most part, lilac-English hybrids bred in the Rockefeller Institute. Individuals of both sexes were employed. All were young adults and each animal was caged separately. The guinea pigs were, for the most part, albino male animals of streptococcus-free stock bred in this Institute. A few guinea pigs from commercial stock were used. Four guinea pigs of one sex were usually caged together. All animals were isolated prior to use and observed for evidence of disease. Only vigorous, healthy animals were employed. Whenever direct comparisons of virulence or titrations of virulence were done, animals of comparable sex, age and weight were selected so that groups would be as nearly as possible alike.

Cultures: The bacteria were grown on Corper's (14) glycerolated egg-yolk medium, adjusted to pH 6.8, except as specifically indicated in individual experiments. Vigorously growing cultures, usually about three weeks old, were used. Suspensions were prepared by grinding a weighed quantity of bacteria, freshly removed from the medium, in a mortar with sterile saline solution so that 1 cc. of suspension contained 1 mg. of bacteria. From this suspension dilutions were prepared to contain the desired quantity of organisms. Considerable care was taken in the preparation of suspensions to avoid clumping and to insure uniform dispersion of bacteria. This was best accomplished by grinding the bacteria first without fluid, then with drop-wise addition of fluid, until several cubic centimetres had been added. The use of phosphate buffers or of a solution of sodium taurocholate did not seem to facilitate dispersion of the organisms. Suspensions were used promptly after preparation. The strains of bacteria will be described under individual experiments.

Inoculations: Intravenous or subcutaneous inoculations were made in the usual manner. Intracerebral inoculations were made by the technique described previously (13), ether or seconal[1] being used for anaesthesia. The latter was administered intraperitoneally in a quantity equal to 20 mg. per kilogram of body weight. With seconal the anaesthesia obtained within twenty minutes following administration was quite adequate for the operative procedure and no postanaesthetic, pulmonary complications were encountered. With two technical assistants, and by employing seconal for anaesthesia, it was found possible to inoculate animals intracerebrally at the rate of about one per minute.

Measurement of results: Largely we have employed the survival time following inoculation as the measure of infectivity and of virulence. When direct comparisons were made on the survival of different groups of animals, the significance of the result was determined by ascertaining the difference in their mean survival and dividing by the probable error of that difference. The result was not considered certainly significant unless the difference was three or more times its own probable error.

Postmortem examinations were done on all animals. Individuals dying from extraneous cause were discarded from further consideration. In many instances microscopical surveys of the lesions were also made.

STUDIES ON NORMAL VARIATIONS IN SURVIVAL AFTER INOCULATION OF A STANDARD DOSE OF ORGANISMS AND TITRATION OF THE MINIMAL LETHAL DOSE OF TUBERCLE BACILLI

In the experiment which follows it may be observed that, with intracerebral inoculation, significant differences in survival do not occur when various groups of carefully selected animals are inoculated with the same dose of the same suspension of organisms. The second half of the experiment was done to determine the end-point of virulence for a given strain, that is, the minimal infective dose.

Five groups each of four male, albino guinea pigs, varying in individual weight from 300 g. to 400 g. and in group weight from 1,420 to 1,440 g., were inoculated intracerebrally with highly virulent bovine tubercle bacilli, strain 39. Each animal was inoculated with the same dose,

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[1] Sodium Propyl-methyl-carbinyl Allyl Barbiturate, Lilly, generously supplied by Eli Lilly and Company, through the courtesy of Dr. G. F. Kempf, Indianapolis City Hospital.

TABLE 1

Normal variations in survival after intracerebral inoculation of 0.000,01 mg. bovine tubercle bacilli—animals of similar weight

NUMBER OF ANIMALS	MEAN WEIGHT	SURVIVAL	
		Mean	PE_M
	g.	days	days
4	355	27.75	1.62
4	360	25.75	4.42
4	355	26.0	1.33
4	357	28.25	2.10
4	355	27.25	1.30
4	487	23.75	3.80

TABLE 2

Variations in survival time due to variations in the dose of organisms injected intracerebrally, and titration of the end-point of infectivity

ANIMAL NUMBER	INOCULATION DOSE	SURVIVED	MEAN SURVIVAL
	mg.	days	days
R 5821*	0.000,01	25	23.75 ± 3.80
R 5822		39	
R 5823		14	
R 5824		17	
R 5825	0.000,001	23	33.75 ± 3.34
R 5826		40	
R 5827		28	
R 5828		44	
R 5829	0.000,000,1	47	35.25 ± 2.95
R 5830		35	
R 5831		33	
R 5832		26	
R 5833	0.000,000,01	S^1	
R 5834		S^2	
R 5835		214	
R 5836		83	

S^1 = Sacrificed 216th day. No tuberculosis.

S^2 = Sacrificed 216th day. Low grade tuberculosis.

* These are serial numbers used in this laboratory over a period of years.

0.000,01 mg., of the same suspension of organisms within the same hour. All succumbed to tuberculosis. Table 1 shows that significant differences in mean survival did not occur among the five groups. A sixth group of slightly older and heavier guinea pigs from the experiment which follows were inoculated at the same time with the same dose of organisms as the five preceding groups. The data on their survival time are included at the bottom of table 1.

In addition to the above, four groups each of four male, albino guinea pigs of individual weights varying between 440 g. and 530 g., and of group weights varying between 1,890 g. and 1,960 g., were inoculated intracerebrally, each group with different doses of the highly virulent bovine strain 39. Each animal of the first group received 0.000,01 mg., each of the second received 0.000,001 mg., each of the third received 0.000,000,1 mg., and each animal of the fourth group received 0.000,000,01 mg. The results of the latter inoculations are shown in, table 2. It will be seen from the data (table 2) that every animal receiving a dose of 0.000,000,1 mg. or more succumbed to the disease. Of the four receiving a dose of 0.000,000,01 mg., two succumbed and two survived; and in one survivor there was unmistakable tuberculosis. The animal which succumbed on the 214th day (table 2) exhibited clinical evidence of disease (loss of weight and paralysis of an extremity) as early as the sixty-first day, was alternately better, then worse, and at autopsy revealed a large, sclerosed tubercle at the base of the brain, together with minor metastatic lesions.

From this experiment it was learned that, with intracerebral inoculation of carefully selected groups of animals, significant differences in survival do not occur in groups receiving the same dose of a given suspension; but significant differences in survival time do occur among groups inoculated with different doses. Finally, it was found that the minimal lethal dose of the bovine strain 39 was between 0.000,000,1 mg. and 0.000,000,01 mg. (see comment in Discussion).

STUDIES ON THE RELATION BETWEEN VIRULENCE OF MYCOBACTERIA AND THE RESISTANCE OF THE HOST

Two female New Zeland red rabbits, weighing 2,720 g. and 2,800 g. respectively, were inoculated intravenously with 0.01 mg. each of bovine tubercle bacilli, strain B39, isolated by the late Dr. Theobald Smith. One rabbit, R 5183,[2] received organisms of a line attenuated by repeated subcultivation on medium adjusted to pH 6.0 (6), while the other, R 5184, received organisms of a line, the virulence of which was maintained through repeated subcultivation on medium adjusted to pH 6.8 (6). X-ray films were made of the thorax of each animal (to show the lung fields) once weekly during the eight weeks following inoculation, and of the surviving animal, R 5183, at monthly intervals for five months additional.

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[2] These are serial numbers used in this laboratory over a period of years.

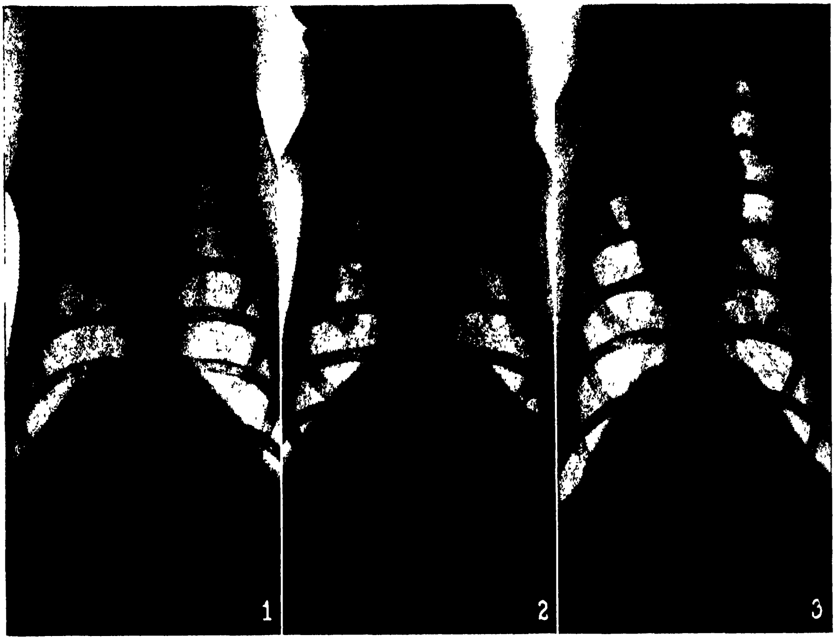

FIG. 1. Roentgenogram of lungs of rabbit R 5183 taken thirty days after inoculation with the attenuated line of bovine strain 39. The lung fields are relatively clear.

FIG. 2. Roentgenogram of lungs of rabbit R 5183 taken fifty-eight days after inoculation with the attenuated organisms. The lung fields still show little abnormality.

FIG. 3. Roentgenogram of lungs of rabbit R 5183 taken one hundred ninety-seven days following inoculation with attenuated organisms. The lungs still are quite clear.

The lung fields of the two animals remained relatively clear during three weeks. In the films taken at the end of four weeks (figure 1), the lungs of R 5183 remained clear, while definite miliary shadows were seen in the lower lobes of R 5184 (figure 4), and there was some haziness of the line of the diaphragm. During the next four weeks the lungs of R 5183 remained clear (figure 2), while the infiltration in the lungs of R

5184 became more pronounced (figure 5) and the plates showed relatively little air-bearing tissue in the lungs. Up to this time the rabbit receiving attenuated organisms, R 5183, had gained 410 g., while R 5184, inoculated with the same dose of fully virulent organisms, had just maintained its preinoculation weight. During the next week the latter lost 600 g. and succumbed on the sixty-fifth day following inoculation. At autopsy massive tuberculous lesions were found, the lungs corresponding to 4.48 per cent of the body weight (0.5 per cent or less being the figure for the normal rabbit (6)). R 5183 remained well and continued to gain weight up to 3,400 g. four and one-half months after the death of the first animal; a final X-ray film of the chest of R 5183 was made and the animal was sacrificed 199 days following inoculation. The X-ray film revealed no pronounced lesions, as shown in figure 3. At autopsy nine small tubercles were found in the lungs, the other viscera showing no lesions.

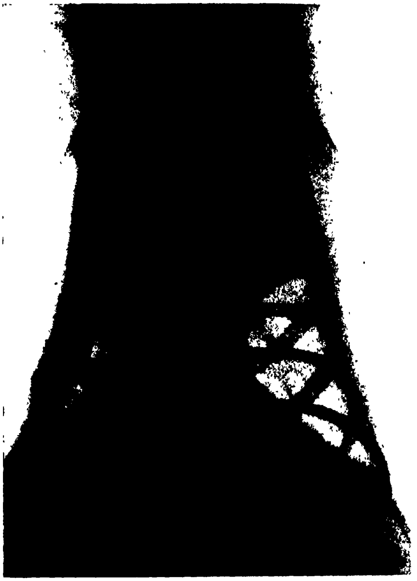

FIG. 4. Roentgenogram of lungs of rabbit R 5184 taken thirty days after inoculation with the highly virulent line of bovine strain 39. The lungs show extensive infiltrations, and the line of the diaphragm is fuzzy.

FIG. 5. Roentgenogram of lungs of rabbit R 5184 taken fifty-eight days after inoculation with highly virulent organisms. The lung fields show massive infiltration throughout and the line of the diaphragm is very irregular. The animal succumbed seven days after the plate was made and showed massive generalized tuberculosis.

This study showed that a normally susceptible host (rabbit) may successfully resist inoculation with attenuated organisms, even in large doses. But a comparable host inoculated with virulent organisms *of the same strain* quickly develops lesions demonstrable by X-ray, and succumbs to massive tuberculous infection. It was interesting to note that lesions were readily demonstrable by X-ray fully a month before there was any loss in weight, and that loss of weight occurred only during the final week of life.

Experiments reported previously (6) had demonstrated the marked differences in virulence of the lines of organisms used in the foregoing experiment, both by intravenous inoculation of rabbits and by quantitative titrations of virulence by intracerebral inoculation of carefully matched groups of guinea pigs.

TABLE 3

Differences in mean survival of groups of guinea pigs inoculated intracerebrally with cultures differing in virulence

INOCULATED WITH STRAIN	DOSE	GROUP RECEIVING ATTENUATED CULTURE SURVIVAL	GROUP RECEIVING VIRULENT CULTURE SURVIVAL	PROBABILITY*	NUMBER OF ANIMALS
	mg.	*days*	*days*		
Bovine B-1	0.01	97.25	34.0	655 to 1 (approximate)	4 per group
Bovine B-1	0.000,1	93.25	54.5	215 to 1	4 per group
H37	0.000,01	36.7	27.6	700 to 1	5 per group

* Odds against chance occurrence of difference of similar order. Difference in mean survival was divided by its own probable error. Probability of the resulting figure taken from Pearl ((15) table 40, page 218).

Smaller differences in virulence may be detected by similar methods and are expressed by differences in survival time of inoculated animals, as shown in the following experiment.

Four groups of young, adult, male guinea pigs were selected, each group consisting of four animals. Individual weights and aggregate weights of the groups were similar. Each animal of one group was inoculated intracerebrally with 0.01 mg. of bovine tubercle bacilli, strain B1 (markedly attenuated), and another group with 0.000,1 mg. of the same culture. Each animal of a third group was inoculated intracerebrally with 0.01 mg. of a line of the bovine strain B1, the virulence of which had been enhanced by animal passage (16); each animal of the fourth group received 0.000,1 mg. of the latter culture.

In addition to the above, two groups, each of five male, albino guinea pigs of matched weight, were inoculated intracerebrally with 0.000,01 mg. of the human strain H37, one group receiving a moderately virulent culture, the second group a culture the virulence of which had been enhanced by animal passage (16). These animals all succumbed to tuberculosis. The mean survival of the six groups, shown in table 3, demonstrates that small or moderate differences in virulence are expressed by differences in survival of the inoculated host. Neither of the bovine B1 cultures was fully virulent, and neither of the human H37 cultures was markedly attenuated, yet significant differences in longevity followed inoculation with the two lines of each. The dose of organisms inoculated into animals included in each horizontal line in table 3 was identical. Thus it is evident that, using the intracerebral route, and inoculating the same dose of different cultures into comparable animals, differences in survival time occur which are proportional to differences in virulence of the cultures.

VARIATIONS IN SURVIVAL TIME DEPENDENT ON THE ROUTE OF INOCULATION

In the concluding study we have investigated the effect of the route of inoculation (portal of entry) on resistance of the host as expressed by survival time. For this analysis animals used as controls in a number of experiments were selected because they had neither been immunized nor treated, because they exhibited no intercurrent illness, because they were of representative stock as regards weight, state of maturity and expected resistance, and finally because each was inoculated with one of two strains of virulent mammalian tubercle bacilli. The analysis includes data from 71 animals inoculated with the human strain H37, and data from 98 animals inoculated with the bovine strain 39 (highly virulent line). In the analysis we have recorded the minimum, maximum and mean survival of groups of animals inoculated with given doses, introduced by various routes. The probable error of the mean survival was determined for each series of animals. These data, together with the number of animals in each series, are recorded in table 4.

Table 4 shows that with either of the two virulent strains there was wide variation in individual survival following subcutaneous inoculation; and with this route of inoculation the mean survival and its probable error were greatest. With intravenous inoculation (bovine 39) the variation in individual survival was less; the mean survival and its probable error were reduced. But with intracerebral inoculation the variation in individual survival was least, the mean survival was also reduced, and the probable error of the mean survival, even with small numbers of animals, was very low. The three groups of animals inoculated by the three different routes with 0.01 mg. of the bovine strain 39 (table 4) vividly illustrate these points. The data also show that minimal doses of virulent organisms introduced intracerebrally cause death in less time, and with less variation in individual survival time than inocula 10,000 times as great introduced intravenously. The prolongation of survival with diminishing doses of organisms introduced by the same route is well shown by the four last groups of animals in table 4.

TABLE 4

Variations in survival time dependent on the route by which the animals were inoculated

INOCULATED WITH STRAIN	ROUTE OF INOCULATION	NUMBER OF ANIMALS	DOSE	SURVIVAL			
				Minimum	Maximum	Mean	PE_M
			mg.	*days*	*days*	*days*	*days*
Human H37	Subcutaneous	15	0.1	32	213	95.5	9.37
	Subcutaneous	24	0.01	55	322	170.2	12.10
	Intracerebral	32	0.000,01	20	46	28.0	0.63
Bovine 39	Subcutaneous*	4	0.01	100	199	144.2	14.05
	Intravenous*	10	0.01	38	103	58.4	3.96
	Intracerebral	8	0.01	13	21	18.1	0.59
	Intracerebral	8	0.000,1	19	29	22.9	0.78
	Intracerebral	55	0.000,01	14†	63†	32.0	0.78
	Intracerebral	13	0.000,001	23	52	35.5	1.54

* Rabbits. All other animals were guinea pigs.

† Only two died under 21 days and only one lived over 49 days.

DISCUSSION

Numerous factors which influence the course of infection caused by mycobateria have recently been discussed by Long (17). He has pointed to the numerous variations in properties of bacteria of the same and of different species and to the variations in susceptibility of individuals of one species, and of different species. Certain of the factors mentioned by Long (17), together with additional ones, have constituted the subjects of the studies here reported. The facts brought out by these experiments point the way to methods of great sensitivity and precision for the bacteriological and immunological investigation of tuberculosis.

Individuals of susceptible stock may effectively resist even intravenous inoculation with large numbers of mycobacteria if those bacteria are low in pathogenic properties (rabbit R 5183, second experiment, as shown in figures 1, 2 and 3). But if the virulence of the organisms is maximal and the host susceptible, death is the result (tables 1, 2 and 4). If the virulent organisms are introduced subcutaneously, resistance of the host has opportunity to express itself, and survival is prolonged. If the virulent organisms are introduced intravenously, lesions are at once set up in visceral organs and resistance of the host is overwhelmed, so that death quickly occurs; and if the inoculation is made intracerebrally, death occurs still more quickly (table 4). With progressive diminution in survival time as influenced by the route of inoculation, there is progressive diminution in variation of survival of different individuals. The resulting decrease in the probable error of the mean survival allows variations in virulence of organisms or in host resistance to be made apparent by relatively small differences in mean survival. The especial significance of these facts is that they maintain even with extremely small inocula introduced intracerebrally. For instance, in the case of the last group of animals in table 4, the mean survival after inoculation of 0.000,001 mg. of organisms was 35.5 ± 1.54 days. Then if one immunized or treated a comparable group of animals (similar number, of similar age, sex, weight, and from the same breeding stock) and inoculated them with the same dose of the same suspension of organisms, and if the variation in survival was such that the probable error of the mean survival was again ± 1.54 days, then a difference of seven days in mean survival would be significant.

The result of the titration experiment (table 2) gave useful information on the infectivity of tubercle bacilli. It is agreed by most investigators that 1 mg. of tubercle bacilli freshly removed from the culture represents about 50,000,000 bacteria. If this be the case, then 0.000,000,1 mg. would be five bacteria, and a dose of 0.000,000,01 mg. would be one bacterium for every second animal inoculated. And if one virulent bacterium could induce fatal disease, then just half those receiving 0.000,000,01 mg. should succumb. The results recorded in table 2 closely approximate this hypothetical result and indicate that the minimal infecting dose of tubercle bacilli introduced intracerebrally is very small, approaching a single virulent bacterium.

SUMMARY

A comprehensive study revealed that very striking differences in survival time occurred, depending on whether tubercle bacilli were inoculated subcutaneously, intravenously or intracerebrally. With the intracerebral route of inoculation the survival time was shortest and individual variation in survival time was least.

Employing the intracerebral route of inoculation and groups of guinea pigs selected with care so that their resistance would be as nearly as possible the same, the following conclusive results were obtained. Comparable groups of animals inoculated with the same dose of the same suspension of organisms did not exhibit significant differences in mean survival time. Comparable groups of animals inoculated with different doses of the same suspension of organisms did exhibit significant differences in survival time. And comparable groups of animals inoculated with the same doses of organisms differing in virulence did exhibit significant differences in survival time.

From these results it may be concluded that resistance, as expressed by survival time following inoculation, may be profoundly affected by the virulence of the organisms, the number of them gaining access to the body, and the route by which they enter the body.

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RESISTANCE TO TUBERCULOSIS

II. VARIATIONS DEPENDENT ON THE AGE OF THE HOST AND UPON RESISTANCE INDUCED BY VACCINATION

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(From the Laboratories of The Rockefeller Institute for Medical Research)

Numerous host-related factors are now known which have an influence on, or in part determine the resistance to tuberculosis. The classic work of Theobald Smith demonstrated variations in resistance dependent on the species. He (1) showed that, while guinea pigs and rabbits are both susceptible to infection with bovine tubercle bacilli, of these two only the guinea pig is susceptible to infection with human type organisms. These observations on the variations in native resistance have been extended to include many animal species and various types of mycobacteria.

Through the work of Wright and Lewis (2), Lurie (3), and Opie and Freund (4), and others, it is now evident that within a given animal species there are genetically transmissible differences in resistance to a given type of tubercle bacillus. Moreover, there is clinical evidence that within one individual there are periodic variations in resistance to tuberculosis, the periods of lowest resistance being puberty, the child-bearing period in women, and the age of the pseudoclimacteric in men.

Regarding the relative resistance of children and of adults, opinions are at variance. Some clinicians hold that resistance is low in childhood and increases with age without regard to altered resistance induced by manifest or latent infection. Others maintain that infants and children are natively more resistant than adults, and that resistance declines with age. An increasing number of workers, led by the Lymanhurst group (5, 6), believe that resistance to primary infection is high at any age, but that resistance to reinfection is much less effective.

The comprehensive experimental studies on antituberculosis vaccination have conclusively demonstrated that resistance can be increased by such procedures, although not without concomitant acquisition of tuberculin hypersensitiveness, the rôle of which in immunity is uncertain. The phenomenon of Koch (7) was probably the first evidence of altered tissue reaction depending on prior experience of the host with the organisms. The various workers who have employed heat-killed organisms, including Römer (8), Petroff (9), Opie and Freund (4) and many others, and the large group who have studied vaccination with BCG have shown beyond doubt that resistance of normal individuals may be at least temporarily increased by active immunization.

In the studies to be reported we have investigated the relative resistance of young and older animals to tuberculosis and have carried out certain experiments on prophylactic vaccination, namely, tests of the efficacy of heat-killed virulent organisms, heat-killed attenuated organisms and living attenuated organisms.

MATERIALS AND METHODS

Animals: Albino guinea pigs, bred at this Institute and from stock free of epizootic streptococcal infection, were used in the first two experiments. In the third experiment the animals used were from commercial stock, all of them free of streptococcal infection as determined by the intracutaneous test of Moen (10). The age, weight and sex of the animals will be discussed under individual experiments. Males and females were separately caged; not more than 4 animals were caged together. All were isolated and observed for evidence of infection prior to any experimental procedure. Only vigorous, healthy animals were used.

Cultures, suspensions and inoculations: The bacteria used for the test inoculations were a highly virulent line of the human strain H37, a moderately virulent line of the same strain, and a highly virulent line of the bovine strain 39 isolated by the late Dr. Theobald Smith (11). These were grown on Corper's glycerolated egg-yolk medium (12) adjusted to pH 6.8. Vigorously growing cultures about three weeks old were used.

Suspensions were prepared by grinding a weighed quantity of organisms, freshly removed from the culture, in a sterile porcelain mortar with drop-wise addition of saline so that 1 cc. of suspension contained 1 mg. of bacteria. From this, appropriate dilutions were prepared to contain the amount of organisms desired for inoculation. All inoculations were done intracerebrally by the method previously described (13), the desired dose of organisms being injected in 0.1 cc. of saline. Anaesthesia for intracerebral inoculations was induced either with ether or by intraperitoneal injection of 0.5 per cent saline solution of seconal,[1] the dose of the latter being 20 mg. per kilogram.

Vaccines: The bacteria used for preparing vaccine were two lines of the bovine strain 39. One, highly virulent, was grown on Corper's medium adjusted to pH 6.8. The second line had lost virulence while under serial subcultivation on Corper's medium adjusted to pH 6.0 (14, 15). Suspensions of these two lines of organisms were prepared in saline so that 1 cc. contained 1 mg. of bacteria. The suspension of attenuated organisms was divided into two portions, one of which was diluted tenfold and reserved for use as living vaccine. The second portion and the suspension of virulent organisms were immersed in a water bath and heated at 60°C. for one hour, care being taken that no organisms escaped full exposure to the heat.

The procedure of the vaccinations will be described in the discussion of the third experiment.

Measurement of results: Variations in resistance were determined by differences in the mean survival time of groups of animals. The significance of the differences was determined by mathematical analysis. The probable error of the mean survival was determined.[2] The difference in mean survival of groups to be compared was determined and the probable error of this difference was calculated. The result was considered significant only when the difference in mean survival was three or more times its own probable error. The probability of such differences was ascertained from Pearl's (16) table 40 (page 218).

At death an autopsy was performed on each animal and surveys were made to determine the extent of macroscopical lesions. Paraffin sections were prepared of brain, lung, spleen, liver, tracheal and cervical lymph nodes of each animal, and stained by Goldner's modification of the Masson trichrome stain (17). In addition, sections of brain, spleen and lung of each animal were stained for tubercle bacilli by the method described by Fuller (18). From the sections microscopical surveys were made to ascertain quantitative and qualitative differences in the lesions of the animals.

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[1] Sodium-Propyl-methyl-carbinyl Allyl Barbiturate, Lilly, generously supplied by Eli Lilly and Company, through the courtesy of Dr. G. F. Kempf, Indianapolis City Hospital.

[2] For all data included in this report the formula for small samples $\left(\sigma = \frac{\sqrt{\Sigma (V-M)^2}}{N-1}\right)$ was used in determining the standard deviation.

VARIATIONS IN SURVIVAL TIME DEPENDENT ON THE AGE OF THE HOST

Although differences in the reaction of young and of older animals to tuberculin have been demonstrated by Freund (19), differences in resistance to tuberculosis dependent on age have not, to our knowledge, been demonstrated. It is well known that young and older animals react dissimilarly to other infectious agents (20). Therefore the following two experiments were designed to ascertain the relative resistance of immature and mature animals to tuberculosis.

First experiment: The animals used for this experiment were of albino stock bred in this Institute. There were 3 females weighing from 840 g. to 1,060 g. and 4 immature males weighing between 120 g. and 180 g. Each of the 7 was inoculated intracerebrally under seconal anaesthesia with 0.001 mg. of the highly virulent bovine strain 39. Seven other animals, including 2 males and one female weighing between 680 g. and 860 g., 2 males and 2 females weighing between 100 g. and 140 g., were inoculated intracerebrally under seconal anaesthesia with 0.001 mg. of the moderately virulent line of the human strain H37. All were allowed to succumb to the disease without other experimental procedure.

Results: Of the 14 animals included in this experiment, 2 died from extraneous cause (nontuberculous pneumonia) and were excluded from further consideration. The average survival of the old animals inoculated with the bovine strain 39 was 21.5 days, as compared with 27.25 days for the young. The average survival of the old animals inoculated with the human strain H37 was 31.3 days; the average for the infant animals was 32.6 days. These differences were suggestive but not conclusive.

This was a preliminary experiment and it was considered probable that the uncertainty of the result was due to the large dose of organisms inoculated. The results, especially in the animals inoculated with the bovine strain 39, seemed suggestive, so that it was decided to conduct a similar, larger experiment using a smaller dose of bacteria.

Second experiment: Three groups of albino guinea pigs were inoculated in this experiment. One group included 3 females and 2 males weighing between 860 g. and 1,040 g., each approximately eighteen months old. A second group included 5 males weighing between 380 g. and 480 g., each approximately three months old. The third group included 5 females weighing from 100 g. to 160 g., each about two weeks old. Each of these 15 animals was inoculated intracerebrally under seconal anaesthesia within the same hour with the same dose of the same suspension of organisms, namely, 0.000,01 mg. of the highly virulent line of human tubercle bacilli, strain H37. All were allowed to succumb to the disease without other experimental procedure.

Results: The mean survival of the three groups of animals is shown in table 1, together with the range of survival and the probable error of the mean survival.

From table 1 it will be seen that the survival time following inoculation was inversely proportional to the age of the animals. The old succumbed first, the middle age group succumbed next, and the younger animals succumbed last. When all three groups were considered there was overlapping of deaths among the groups. But, disregarding for the moment the animals of the middle age group, all animals of the older group succumbed before the time of the first death among the infant animals. Differences in survival time, of the order observed here (table 1) have been shown (21) not to occur when animals of similar age and weight are inoculated with the same dose of the same suspension of organisms. But the differences recorded in table 1 are even more striking when it is noted that the dose of organisms for the young animals, expressed as mg. of bacteria per kilogram body weight, was 7.7 times the dose given to the old and 3.3 times the dose given to the group of middle age. It is therefore apparent that resistance to a given inoculum varies with age and is inversely proportional to it.

TABLE 1

Variations in survival after intracerebral inoculation of 0.000,01 mg. human tubercle bacilli—animals of different age and weight

GROUP	NUMBER OF ANIMALS	MEAN WEIGHT	SURVIVAL			
			Minimum	Maximum	Mean	PE_M
		grams	*days*	*days*	*days*	*days*
A	5	988	19	27	23.8	0.89
B	5	424	22	33	27.6	1.22
C	5	128	32	39	35.8	0.87

Difference B − A, 3.8 days is 2.5 × its PE. Odds 9.9 to 1.
Difference C − B, 8.2 days is 5.5 × its PE. Odds very high.
Difference C − A, 12.0 days is 9.6 × its PE. Odds very high.

Microscopical studies of the lesions which developed in these three groups of animals showed no essential differences in the type of lesions, nor in their distribution. All animals exhibited acute tuberculous meningoencephalitis and metastatic visceral tuberculosis. But both the local and metastatic visceral lesions were the more extensive in the young animals, and least extensive in the oldest animals. From the histological studies it appeared that the infection proceeded in similar fashion and at a similar rate in young and older animals, but that less tuberculosis was necessary to cause death in the older animals. Lesions were the more extensive in the youngest animals because they lived longest.

STUDIES ON RESISTANCE INDUCED BY VACCINATION

Several experiments on antituberculosis vaccination have been carried out in this laboratory, employing methods similar to those in the experiment which follows. The results of all were in agreement.

The purpose of the experiment was to compare the protective properties of living attenuated, and killed virulent organisms, and to ascertain if the immunizing principle of attenuated organisms is thermolabile.

Third experiment: Vaccines consisting of suspensions of (*a*) living attenuated organisms, (*b*) heat-killed attenuated organisms, and (*c*) heat-killed virulent organisms, were prepared as described under Materials and Methods. The suspension of living organisms contained 0.1 mg. of bacteria per 1 cc. The suspension of heat-killed organisms contained 1.0 mg. per 1 cc. The vaccines were injected daily by the subcutaneous route on the right side. The daily dose was 0.2 cc. and five doses were given, so that those injected with living attenuated organisms received a total quantity of 0.1 mg. of organisms, and the two series injected with heat-killed bacteria received a total quantity of 1.0 mg. per animal.

Fifty-six male guinea pigs obtained from commercial stock were isolated and observed for a week. During this time each was tested intracutaneously for streptococcal infection by the method of Moen (10) with extract generously supplied by him. Three animals were found to be reactors and were discarded. Each of the 53 remaining animals weighed between 360 g. and 420 g. at the start of the experiment. The animals were first subdivided into four groups. One group of 13 served as controls and were not vaccinated; a group of 11 were vaccinated with heat-killed virulent organisms; a group of 11 were vaccinated with heat-killed attenuated organisms; and a group of 18 with living attenuated organisms. Of the latter series (vaccinated with living attenuated organisms) 4 individuals were reserved without further experimentation to ascertain whether the living vaccine produced progressive disease.

Following the vaccinations the animals of each series gained weight, with a single exception.[3] The gain in weight was approximately the same in the four series of animals and bore no relation to experimental procedures.

Forty-four days following the last vaccinations the four series of animals were subdivided as shown in table 2. Approximately one-third of each series was included in each subgroup. Each animal of one subgroup of controls and each animal of one subgroup of the three series previously vaccinated were then inoculated intracerebrally with 0.01 mg. of highly virulent bovine tubercle bacilli, strain 39. One subgroup of the controls and one subgroup of each vaccinated series were similarly inoculated with 0.000,1 mg. of the same strain of organisms. The remaining one-third of the animals, including one subgroup of the non-vaccinated controls and one subgroup of each vaccinated series, were inoculated intracerebrally with 0.000,001 mg. of the same strain of bacilli. These inoculations were done under ether anaesthesia. Eight of the subgroups included 4 animals each, two included 3 animals each, one included 5 animals and one included 6 animals, as shown in table 2. Following the test inoculations the animals were allowed to succumb without other experimental procedure.

At death a necropsy was done on each animal, and gross surveys of the lesions were made. Microscopical sections of the tissues were made as described under Materials and Methods, and the four series of animals were compared as regards type, distribution and extent of lesions.

Results: The 4 animals which were vaccinated with the living attenuated organisms, but not inoculated with virulent organisms, remained well and gained weight. One hundred and ninety-five days following the last dose of vaccine each was sacrificed to ascertain whether the living bacteria used as vaccine had induced disease. Only one of the 4 exhibited lesions at necropsy; these consisted of a few small hard nodules in the spleen. Microscopically these lesions were fibrotic tubercles. In one of them a single acid-fast bacillus was found. This animal showed no other microscopical lesions, and the remaining 3 animals showed no lesions whatever. This result indicated that the organisms were indeed attenuated, as we had previously observed (15).

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[3] This animal, one of those vaccinated with killed virulent organisms, and subsequently inoculated with the largest test dose, did not gain weight at any time. However, it did survive the test inoculation for a period slightly longer than the mean for the group, and the pathological findings were in no wise different from those of experimental mates.

Table 2 shows the result of the experiment on prophylactic vaccination. The survival time of each animal following test inoculation and the mean survival time of each group is recorded.

The very slight differences in mean survival time of two of the vaccinated groups and the control group inoculated with 0.01 mg. of organisms were not significant. The differences between the mean survival time of controls and of each vaccinated group receiving 0.000,1 mg. were significant by direct analysis; and in the groups receiving 0.000,001 mg. of organisms the differences proved also to be significant.[4] Moreover, the one vaccinated animal (table 2) which was sacrificed to terminate the experiment, survived the test inoculation.

TABLE 2

Survival time of each individual and mean for each group, in three series of vaccinated animals and in their nonvaccinated controls

TEST INOCULATION DOSE	CONTROLS NONVACCINATED		VACCINATED WITH HEAT-KILLED VIRULENT TUBERCLE BACILLI		VACCINATED WITH HEAT-KILLED ATTENUATED TUBERCLE BACILLI		VACCINATED WITH LIVING ATTENUATED TUBERCLE BACILLI	
	Survival time in days following inoculation							
	Individual	Mean	Individual	Mean	Individual	Mean	Individual	Mean
mg. 0.01	18 18 20 20	19.0	19 20 26	21.6	15 21 25	20.3	Died overnight	
0.000,1	19 20 21 21	20.25	30 33 35 38	34.0	31 34 46 47	39.5	28 37 37 57	38.0
0.000,001	29 33 35 45 52	38.8	35 43 57 183*	79.5	37 37 41 65	45.0	37 39 45 47 119 145	72

* This animal was sacrificed when in good health to terminate the experiment. No macroscopical lesion was found. In sections a single minute "hard" tubercle was found in the cerebellum. No tubercle bacilli were found in the lesion.

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[4] In the latter instance the variability was so great, and consequently the probable error of the mean survival was so great, that direct analysis did not reveal the significance of the result. However, this was brought out by arranging each vaccinated group with the control

Thus, while only one animal survived as result of the vaccinations, survival time was prolonged in all the vaccinated groups receiving either 0.000,1 mg. or 0.000,001 mg. But there was no demonstrable protection in the groups inoculated with 0.01 mg. Indeed the group vaccinated with living attenuated organisms and inoculated with 0.01 mg. succumbed during the night following inoculation (table 2). Similar results were observed by Soper and Dworski (23) and it is clear that the allergy induced by vaccination may have a detrimental effect if the superinfecting dose is large.[5]

The histological studies of animals included in this experiment revealed additional evidence of a protective effect. All of the control animals, even those receiving the 0.000,001 mg. dose of virulent organisms, exhibited generalized tuberculous meningoencephalitis, as shown in figures 1 and 2. Figure 1 is a photograph at low magnification of the brain of the control animal, R 5170, inoculated with 0.000,001 mg. Death occurred on the forty-fifth day. Like its experimental mates this animal showed extensive meningitis, and lesions in remote viscera (spleen, liver, etc.). Figure 2 from the same animal shows, at higher magnification, the character of the lesions shown in figure 1. The exudate is composed of epithelioid cells, neutrophiles and monocytes, and there is a good deal of caseation.

In contrast to these findings many of the vaccinated animals, especially those which lived the longer, exhibited no meningitis, or at least only localized inflammation. Instead, there was present in the brain, and usually at the base on the side of inoculation, a localized tubercle of greater or lesser size. Such a lesion is shown at low magnification in figure 3 and at higher magnification in figure 4. There is no caseation. At the lower margin of the lesion there is a closely packed mass of lymphocytes. The lesion itself is composed almost wholly of epithelioid cells. These photographs were made of the brain of guinea pig R 5161, of the group vaccinated with heat-killed virulent organisms and

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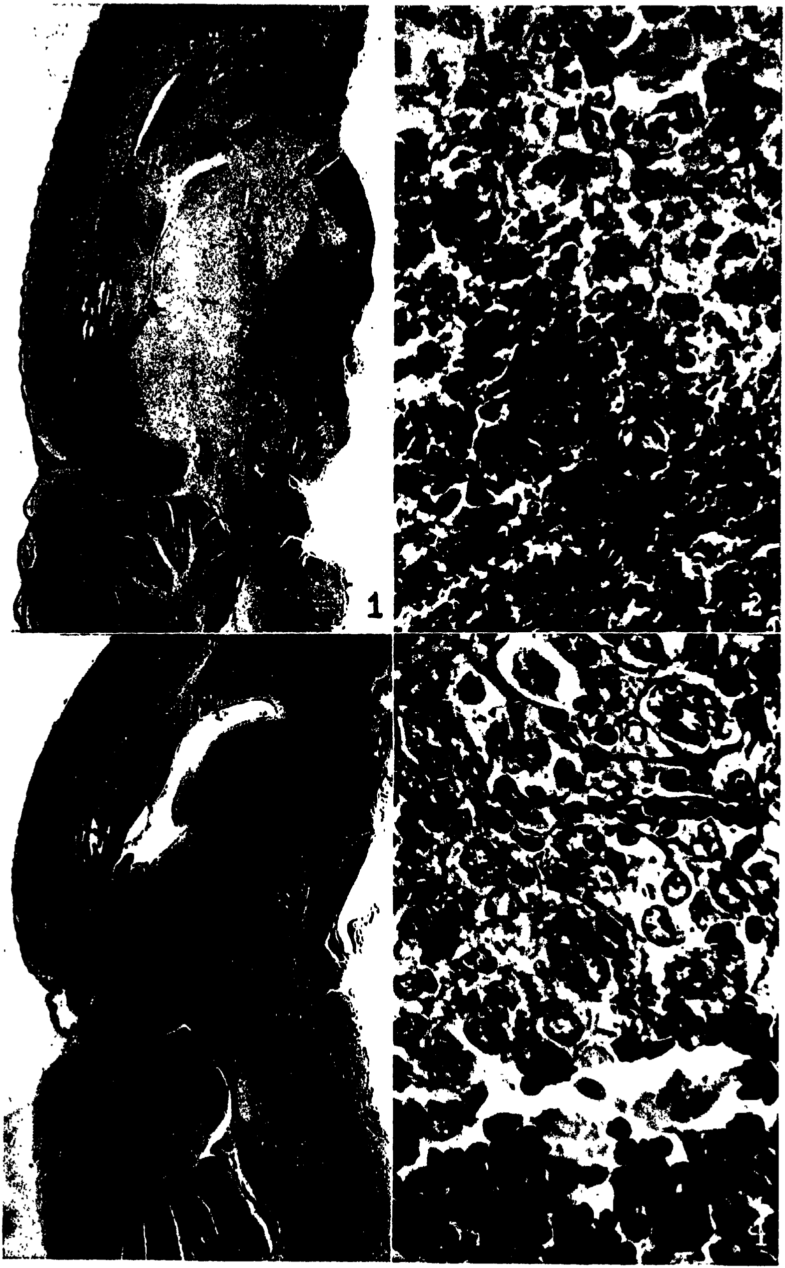
as one series, and determining the standard deviation. Then, employing the method shown by Davenport and Ekas ((22) page 21) the significance of the result was shown by the fact that one animal in each vaccinated group could be rejected as an extreme variate. Thus, while the controls were a normal series, each of the vaccinated groups receiving 0.000,001 mg. contained individuals showing extreme variation in survival time, and this extreme variation was undoubtedly a result of partial protection afforded by the vaccination.

[5] This effect is not characteristic alone of intracerebral inoculation. In other studies we have vaccinated rabbits to produce immune serum for serological work. Animals previously vaccinated, then allowed to rest, and again injected with as little as 0.3 mg. of killed bacilli, frequently succumbed during the next twelve to twenty-four hours.



FIGS. 1–4

inoculated with 0.000,001 mg. of bovine strain 39. Death in this animal occurred forty-three days following inoculation. By comparing figure 3 with figure 1 it may be seen that the lesions of the vaccinated animal (figure 3) were quite localized. And figure 4 shows that the local lesion differed histologically from that in the control, figure 2. A further difference between such vaccinated animals and their controls was that the vaccinated guinea pigs exhibited little or no visceral tuberculosis, while the controls invariably showed lesions in spleen, liver and lymph nodes. Thus, in animals which developed appreciable immunity or resistance as a result of vaccination there was concomitantly developed a power to localize the virulent organisms and to prevent their spread to remote parts of the body. The immune reaction, however, was not adequate to destroy the bacilli *in situ*, and there they remained, perhaps multiplying slowly and eventually causing death either from paralysis and inanition or by involvement of some vital cerebral centre.

DISCUSSION

The fact that resistance to tuberculosis varies with age, and that this variation is characterized by a progressive decline in resistance with advancing age, must be taken into account in any experiments in which the attempt is made to influence the course of the disease either by treatment or by prophylactic measures. Especially is this true when the criterion of effect is the survival time following inoculation. In large groups of animals inoculated by the intracerebral route, very small

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FIG. 1. Saggital section of left cerebral hemisphere of guinea pig R 5170, a nonvaccinated control inoculated intracerebrally with 0.000,001 mg. of the bovine strain 39. Animal succumbed on the forty-fifth day following inoculation. Note very extensive meningitis and involvement of brain substance at the base, and lesions in cerebellum. Masson trichrome stain. 5.5 ×

FIG. 2. Representative area from same section as fig. 1, showing character of the meningeal reaction. Lower portion of photograph shows border of a caseous focus. Elsewhere the cellular reaction is complex and shows many neutrophiles. There is much cellular degeneration. Masson trichrome stain. 720 ×

FIG. 3. Saggital section of left cerebral hemisphere of guinea pig R 5161, which was vaccinated with heat-killed virulent organisms and inoculated intracerebrally with 0.000,001 mg. of the bovine strain 39. Death occurred on the forty-third day following inoculation. Note circumscribed lesion at base of brain, minimal meningitis and absence of cerebellar lesions. Masson trichrome stain. 5.5 ×

FIG. 4. Higher magnification of the lesion seen at the base of the brain in fig. 3. Note absence of caseation. The lower border of the lesion is heavily infiltrated with lymphocytes. The tubercle is made up almost wholly of epithelioid cells which show no degenerative change. Masson trichrome stain. 720 ×

differences in mean survival time may be highly significant, as we have shown (21). Then if age differences be not taken into account, an apparent beneficial effect might prove to be a false one; or a truly beneficial effect might be masked by differences in resistance due to age. These facts emphasize the great importance of careful selection of experimental hosts, with especial regard to the age and weight.

The greater resistance of the young individual to tuberculosis which we have observed experimentally is in accord with the experience of certain clinicians. Although the young guinea pig lacks the power which many believe to be indigenous in children—the power to heal a primary infection—and may therefore be considered less resistant than a young human being, resistance of the older guinea pig is still less, and significantly so.

As regards resistance induced by vaccination, our experiments confirm those of Soper and Dworski (23) that enhanced resistance may be so induced, but our results do not indicate the better protection with living attenuated organisms. Instead, we are led to believe, as do Opie and Freund (4) and as did Petroff (9), that the result to be obtained with killed virulent organisms is equally good. However, the hypersensitiveness induced by vaccination cannot be ignored, and we observed, as did Soper and Dworski (23), that a large infecting dose may cause early death in a sensitized individual.

It may well be that our results and those of Soper and Dworski (23) offer a clue to the old controversy as to the relation of allergy and immunity. It is generally agreed by all participating in this controversy that the immunized resistant host is allergic; to certain workers this has indicated a direct relationship; but others have maintained that immunity is a thing apart from allergy. Certainly it is true that an allergic host may not be unusually resistant (24). If, then, a certain quantity of allergen is necessary to evoke the allergic response, the sensitized animal which received a small test inoculation might demonstrate the immune response without exhibiting allergic reaction. But if the inoculating dose were sufficient to contain the necessary quantity of allergen, then the allergic reaction might, by explosive violence, quickly kill the host. In such case the allergic state would be detrimental only when the superinfecting dose was larger, but in any case might bear no closer relation to immunity than does allergy in a host which is not immune (24).

However this may be, in clinical practice the dose of organisms with which man becomes infected cannot be controlled as in the laboratory; and it follows that occasional untoward results might be expected in any large program of antituberculosis vaccination. Moreover, as we have recently shown (25) attenuated organisms may regain virulence through host to host passage. Therefore, in our opinion, vaccination by any method now known is not without its dangers; and the potential dangers are greater in the case of vaccine composed of living mycobacteria.

There is a possibility that the immunizing substance, or substances, contained in the organisms may be separate and distinct from those which induce allergy. Should this be the case, isolation of the immunizing antigen would eliminate the difficulties just discussed.

The capacity of the resistant vaccinated animal to localize bacteria inoculated intracerebrally, together with the absence of meningitis and of visceral lesions, leads to another interesting speculation. Lesions similar in kind have been observed (25) in animals surviving long after intracerebral inoculation with attenuated organisms. This gives rise to the question whether the virulent organisms injected into immunized animals might not have suffered a decline in virulence while residing in the resistant host. Such a change could be detected experimentally but opportunity to investigate this point has not been available.

The results recorded in table 2 do not indicate that the immunizing antigen in tubercle bacilli is thermolabile.

SUMMARY

Variations in resistance to tuberculosis depending on the age of the host have been studied by sensitive methods. Young individuals proved to be the more resistant, and there is a progressive decline in resistance with advancing age.

Resistance induced by vaccination was expressed by greater survival time of vaccinated individuals following intracerebral inoculation of small doses of virulent organisms. The resistant host has a capacity to localize the organisms and restrict them to the brain, but there the organisms survive. An occasional vaccinated animal may survive the virulent inoculation.

Hypersensitiveness accompanying the enhanced resistance may cause death if the infecting dose is large.

Certain implications of the work are discussed.

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COMPARISON OF THE EFFECT OF AN HOMOLOGOUS TUMOR MATERIAL AND THE DURAN-REYNALS FACTOR ON TUMOR GROWTH[1]

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Frozen and anaerobically preserved Brown-Pearce tumor tissue has been found to contain a filtrable material which, when injected parenterally two weeks before tumor inoculation, renders the inoculated animal more susceptible to the growth of this tumor (1). This increased susceptibility is expressed in a greater incidence, volume, and number of primary and metastatic tumors, and in a greater mortality and decreased longevity from tumor growth. The material had no demonstrable effect on certain transplanted mouse tumors, nor have similarly prepared materials from the mouse tumors been effective in altering the growth of the Brown-Pearce tumor in rabbits (2).

Since this material from the Brown-Pearce tumor has the power of augmenting the invasion of the body by tumor cells and of enhancing tumor growth, it seemed wise to compare its action with that of the factor described by Duran-Reynals (3), which has the power of increasing the invasion of the body by animal poisons, bacteria, and viruses (4, 5). This "spreading factor," as it is called, has been discovered in the venom of poisonous snakes, in the bodies of poisonous insects such as mosquitoes, spiders, etc., in virulent bacteria, in normal mammalian tissues especially those of the testicle, and in malignant mammalian tumors. Its presence in malignant mammalian tumors has been discussed by Duran-Reynals and Stewart, by Tanzer, by Prime and Haagensen (6), by Walker (11), and by McClean (7). It has been found in only small amounts in benign tumor tissues, whereas malignant tissues contain large amounts comparable with those found in normal testicle. It seemed necessary, therefore, to determine whether the spreading factor described by Duran-Reynals and by McClean could possibly account for the action of the homologous material obtainable from the Brown-Pearce tumor. To this end four experiments were carried out testing the effect of the Duran-Reynals factor on the growth of the Brown-Pearce tumor, when used in a manner similar to that employed for the homologous material.

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[1] This work was largely supported by a grant from the International Cancer Research Foundation. An abstract of this material was presented before the American Society for Experimental Pathology in Memphis, 1937.

EXPERIMENTAL STUDIES

Since testicle extract has been found by Duran-Reynals to be one of the most fertile sources of the factor, experiments were done using testicle extract prepared in the laboratory from normal rabbits and bull testicle extract kindly sent to the author by Dr. Duran-Reynals (Tables I and II).

Experiments 1 and 2: Twenty-four seemingly healthy gray-brown rabbits, young adult males, obtained from a single dealer, were divided into four equal groups of 6 each. The groups were matched as closely as possible with regard to the appearance, weight, and body build of the animals. On Feb. 21, 1931, the 6 rabbits of Group A (Experiments 1 and 2) were injected in the right testicle with 0.3 c.c. of a saline emulsion of homologous tumor material prepared in the usual manner from Brown-Pearce rabbit tumor tissue (8). The 6 rabbits of Group E, Experiment 1 (E1 to E6 inclusive), were inoculated on the same day with 0.3 c.c. of a saline emulsion of normal rabbit testicle. The rabbits of Group E, Experiment 2 (E7 to E12 inclusive), were also injected, on the same day, with 0.3 c.c. of a saline emulsion of normal rabbit testicle which had been frozen, embedded in paraffin, and preserved at 28° F. for a period of two weeks, this being the method used in preparing the homologous tumor material. The 6 rabbits of Group C (Experiments 1 and 2: C1 to C6 inclusive) were controls and were not inoculated at this time. Two weeks later all the rabbits seemed healthy and no evidence of the original injection into the testicle was detected. Each of the 24 then received an injection into the left testicle of 0.3 c.c. of fresh saline emulsion of living Brown-Pearce tumor tissue. The animals were examined twice weekly thereafter. Animals dying were autopsied. At the end of the usual two months' observation period surviving animals were killed with air, and careful post-mortem examinations were carried out by the routine worked out in previous experiments (8).

Experiment 3: Eighteen young adult male Chinchilla rabbits, seemingly in good health, were obtained from one dealer and divided into three groups of 6 each. On April 11, 1936, 0.3 c.c. to 0.55 c.c. of a fresh bull testicle extract, sent to the author in ampules by Dr. Duran-Reynals, was injected into the right testicle of each of the 6 animals of Group E as follows: 0.3 c.c. E13; 0.35 c.c. E14; 0.40 c.c.

E15; 0.45 c.c. E16; 0.5 c.c. E17; 0.55 c.c. E18. The 6 rabbits of Group A were injected with 0.3 c.c. of a saline emulsion of homologous material prepared from rabbit tumor tissue frozen and anaerobically preserved. The 6 rabbits of Group C (C7 to C12 inclusive) were controls and were not injected on April 11. Two weeks later, on April 25, 1936, the 18 rabbits comprising the three groups were injected in the right testicle with 0.3 c.c. of a saline emulsion of living fresh Brown-Pearce rabbit tumor. Observations and autopsies were carried out as in the first and second experiments.

Experiment 4: Thirty-four young adult male Chinchilla rabbits, seemingly in good health, were obtained from a single dealer, and divided into two groups of 17 each. On May 21, 1936, 0.2 c.c. of bull testicle extract was injected into the right testicle of the rabbits of Group E (Experiment 4; E19 to E35 inclusive). This extract was received from Dr. Duran-Reynals in an ampule on April 8, 1936, and had been kept in the ice-box since that time. The second group of 17 rabbits (C13 to C29) were controls and not injected on that day. Several days after injection one of the rabbits, E35, died of a lung infection, leaving only 16 in the group. On June 6, 1936, at the end of two weeks, the remaining rabbits were in apparent good health and showed no evidence of the previous injection. All 33 were then injected in the left testicle with 0.3 c.c. of fresh, living, Brown-Pearce rabbit tumor tissue. Observations and autopsies were conducted as in the previous experiments.

TABLE I

Data on Materials and Methods

Experiment	Group and Number of Animals	Preliminary Treatment			Inoculation of Brown-Pearce Tumor in Left Testicle	
		Material Injected in Right Testicle	Dosage (c.c.)	Date	Dosage (c.c.)	Date
1	E6	Saline emulsion of fresh rabbit testicle	0.3	Feb. 21, '31	0.3	Mar. 7, '31
2	E6	Saline emulsion of frozen and preserved rabbit testicle	0.3	Feb. 21, '31	0.3	Mar. 7, '31
1 and 2	C6	Nothing	—	Feb. 21, '31	0.3	Mar. 7, '31
1 and 2	A6	Homologous tumor material	0.3	Feb. 21, '31	0.3	Mar. 7, '31
3	E6	Bull testicle extract	0.3–0.55	Apr. 11, '36	0.3	Apr. 25, '36
	C6	Nothing	—	Apr. 11, '36	0.3	Apr. 25, '36
	A6	Homologous tumor material	0.3	Apr. 11, '36	0.3	Apr. 25, '36
4	E17	Bull testicle extract	0.3	May 21, '36	0.3	June 6, '36
	C17	Nothing	—	May 21, '36	0.3	June 6, '36

TABLE II

Experimental Results

Duran-Reynals Factor Group						Control Group					
Rabbit	PT (c.c.)	MT (c.c.)	MF (no.)	Long (days)	Mort	Rabbit	PT (c.c.)	MT (c.c.)	MF (no.)	Long (days)	Mort
Experiment 1											
E1	0	0	0	62	R	C1	11	96	16	58	D
E2	14	63	14	54	D	C2	15	22	4	62	I
E3	0	0	0	62	R	C3	11	102	21	55	D
E4	17	62	15	60	D	C4	15	82	13	62	I
E5	0	0	0	62	R	C5	35	192	25	38	D
E6	0	0	0	62	R	C6	0	0	0	62	R
Experiment 2											
E7	0.5	0	0	61	I	C1	11	96	16	58	D
E8	0	0	0	62	R	C2	15	22	4	62	I
E9	20	165	25	45	D	C3	11	102	21	55	D
E10	15	3	2	62	I	C4	15	82	13	62	I
E11	Died: Lung abscess					C5	35	192	25	38	D
E12	9	2	1	62	I	C6	0	0	0	62	R
Experiment 3											
E13	9	33	16	35	D	C7	6	50	14	59	D
E14	7	2	3	60	I	C8	6	133	23	43	D
E15	18	12	5	60	I	C9	7	4	3	39	D
E16	11	6	3	60	I	C10	0	0	0	60	R
E17	9	1	1	26	D	C11	0	0	0	60	R
E18	14	21	9	54	D	C12	0	10	9	59	D
Experiment 4											
E19	4	59	21	28	D	C13	0	0	0	62	R
E20	0	0	0	62	R	C14	0	0	0	62	R
E21	10	29	12	40	D	C15	0	0	0	62	R
E22	0	0	0	62	R	C16	2	1	3	34	D
E23	1	0	0	62	I	C17	4	5	9	39	D
E24	6	35	27	31	D	C18	8	16	12	61	D
E25	10	96	24	45	D	C19	0	0	0	62	R
E26	5	1	3	62	I	C20	3	7	10	40	D
E27	16	30	23	37	D	C21	13	23	6	62	I
E28	16	10	8	32	D	C22	8	53	20	39	D
E29	0	0	0	62	R	C23	0	0	0	62	R
E30	0	0	0	62	R	C24	0	0	0	62	R
E31	4	7	5	62	I	C25	0	0	0	62	R
E32	8	48	18	44	D	C26	0	98	14	62	I
E33	34	97	14	40	D	C27	0	0	0	62	R
E34	0	0	0	62	R	C28	0	32	11	30	D
						C29	0	0	0	62	R

E = Experimental. C = Control. PT = Primary tumor. MT = Volume of metastatic tumor. MF = Metastatic foci. Long = Longevity after inoculation. Mort = Mortality. D = Died from tumor. R = Resistant. I = Indefinite, having tumor foci but alive at the end of the two-month period.

In Experiments 2, 3, and 4 the primary tumors seemed to appear earlier and to grow to a larger size in a shorter period of time in the animals treated with the testicle extracts than in the untreated controls. This observation was similar to that for groups treated with homologous material. There was a difference, however, in that the tumors in animals treated with homologous material continued to grow and extend, whereas those in animals treated with testicle extract seemed to lose their growth capacity after four weeks, and at post-mortem examination no difference, so far as tumor volume was concerned, could be detected between the controls and the treated rabbits. Unfortunately, the records of measurements of primary tumor growth during life were lost, and no exact figures, except those obtained post mortem, are available.

The results for animals treated with testicle extract and for the control animals not so treated are presented in Table II. It will be seen that one group served as a control for both Experiment 1 and Experiment 2. For accuracy in statistical calculation this group is considered twice in the table.

The results for the animals treated with the homologous material were similar to those noted in previous experiments in that there were more and larger primary tumors, more and larger metastatic tumors, a greater mortality, and a decreased longevity as compared to the control animals. Since they are not pertinent here, the results of these experiments will be considered only in the discussion.

Analysis of Results

Incidence of Primary Tumors: In Table III an analysis of the incidence of primary tumors was made with the Chi-square test (9). It will be seen that there was no significant variation between the number of primary tumors in the control series and the series receiving the Duran-Reynals factor.

Volume of Primary Tumors: The average volume of the primary tumor at post-mortem examination was 6.77 c.c. among the controls and 7.79 c.c. among the animals treated with the Duran-Reynals factor. The difference, 1.2 ± 2.1 c.c., is not significant ($t = 0.6$; $P = 0.55$) (9). Among the animals treated with the testicle extract the volume of the primary tumor averaged 11.17 c.c., as compared to

11.85 c.c. for the controls. The difference, 0.68 ± 2.5 c.c., is obviously not significant.

Incidence of Metastatic Tumors: Table IV presents an analysis of the incidence of metastatic tumor among animals with primary tumor as determined at post-mortem examination. By the Chi-square test there was found no difference in the incidence of metastatic tumors in the two series.

TABLE III

Incidence of Primary Tumors

	Actual Incidence			Expected Incidence		
	+	–	Total	+	–	Total
Duran-Reynals Factor Series	23	10	33	20.9	12.1	33.0
Control Series	20	15	35	22.1	12.9	35.0
Total	43	25	68	43.0	25.0	68.0

$X^2 = 1.1$. $n = 1$. $P = 0.75$. Not significant.

TABLE IV

Incidence of Metastatic Tumor Among Animals with Primary Tumor at Post-Mortem Examination

	Actual Incidence			Expected Incidence		
	+	–	Total	+	–	Total
Duran-Reynals Factor Series	21	2	23	21.9	1.1	23.0
Control Series	20	0	20	19.1	0.9	20.0
Total	41	2	43	41.0	2.0	43.0

$X^2 = 1.83$. $n = 1$. $P = 0.18$. Not significant.

Volume of Metastatic Tumors: Estimated on the basis of animals with metastases the volume of the metastatic tumor averaged 37.2 c.c. in the Duran-Reynals factor series and 64.5 c.c. among the controls. The difference, 27.3 c.c., was quite large and very suggestive, but the error was 15.3 c.c. and was not significant ($t = 1.78$, $n = 41$, $P = 0.75$).

Number of Metastatic Foci: As in previous experiments, the animal body was divided into arbitrary areas, 50 in number. The presence of one or more metastatic tumor nodules in one of these areas was considered to represent a single tumor focus. On the basis of animals with metastases the Duran-Reynals factor series averaged 11.9 metastatic foci and the control series 12.1 metastatic foci. The difference, 0.28 ± 2.6, is not significant ($t = 0.11$, $n = 43$, $P = 0.9$).

Mortality: In Table V an analysis of the mortality by the Chi-square test is presented. No significant variation between the control and experimental series was noted in this respect.

Longevity: On the basis of the number of days of survival among animals with metastatic tumors the longevity among the controls was 50.8 days and in the experimental series 47.6 days. The difference, 3.2 ± 3.7, was not significant ($t = 0.84$, $n = 43$, $P = 0.4$).

TABLE V

Mortality from the Brown-Pearce Tumor

	Actual Mortality				Expected Mortality			
	R	D	I	Total	R	D	I	Total
Duran-Reynals Factor Series	10	14	9	33	11.2	14.5	7.3	33.0
Control Series	13	16	6	35	11.8	15.5	7.7	35.0
Total	23	30	15	68	23.0	30.0	15.0	68.0

R = Resistant, negative at necropsy. D = Died from tumor. I = Indeterminate, living but showing tumor at the end of two months.

$X^2 = 1.0$. $n = 2$. $P = 0.8$. Not significant.

DISCUSSION

From the analysis of results it will be seen that there were no significant differences between the control series and the series receiving the Duran-Reynals factor as to the incidence or volume of the primary tumors, as to the incidence, number, or volume of the metastatic tumors, or in the longevity and mortality after inoculation. There is thus no evidence that the action of the Duran-Reynals factor can explain the effect of the homologous tumor material, since the latter seems to enhance every phase of primary and metastatic tumor growth enumerated above.

Since the number of metastatic foci per animal with metastases has been found to be the most reliable criterion of the action of the homologous material, it was thought desirable to present graphically a summary of the present and previous experiments on this point. In Fig. 1, Column 6 represents the number of metastatic foci (18.6) per animal inoculated with homologous material in Experiments 1, 2, and 3 of this series; Column 2 represents the number of metastatic foci among the control animals in the present experiments, and Column 4 represents the number of metastatic foci among animals treated with the Duran-Reynals factor. Column 1 represents the control series and Column 5 a series receiving homologous material described in an earlier publication (8). Column 3 represents the number of metastatic foci among animals with metastases in a total of 367 control rabbits inoculated in various other experiments during the past ten years, and Column 7 the total experience to date (16 experiments, 94 animals) with animals treated with the unfiltered homologous material from the Brown-Pearce tumor. It will be seen that the number of metastatic foci in the present experiments is in accordance with previous experience. Since the number of metastatic foci is one of the most reliable criteria on the action of the homologous material, there is no evidence that that action can be explained as an effect of the Duran-Reynals factor.

The mortality-longevity relationship is also a reliable criterion of the action of the homologous material. In Fig. 2 the total experience to date with homologous material and the respective control animals is presented. The mortality-longevity relationship in the animals receiving the Duran-Reynals factor and the control series is also presented in Fig. 2. It will be seen that the mortality curve for the Duran-Reynals factor series coincides with that of the several control series, and that it is significantly different from that for the homologous material.

Experiments now in progress indicate that a crude emulsion of the homologous material from the rabbit tumor can be inactivated at 55° C. (10). The homologous rabbit tumor material has not been effective so far in stimulating the growth of certain mouse tumors nor have similarly prepared extracts from the mouse tumors been effective in stimulating the growth of the Brown-Pearce tumor (2). The Duran-Reynals factor, on the other hand, has been obtained in a wide variety of vertebrate and invertebrate tissues and in bacteria and does not seem to be species or tissue specific in its origin, source, or action.

The observation that testicle extract may enhance the primary growth of the Brown-Pearce tumor, as in Experiments 2, 3, and 4 of this series, is not new. Walker in 1935 described an early 200 per cent increase in the local growth of the rabbit tumor in the skin and in the testicle following the intravenous inoculation of testicle extract daily after tumor inoculation (11). The suggestion in the present work is the possibility that the testicle extract may exert an effect on tumor growth in animals inoculated two weeks after its injection. If this is true, either the material persists in the body for two weeks or

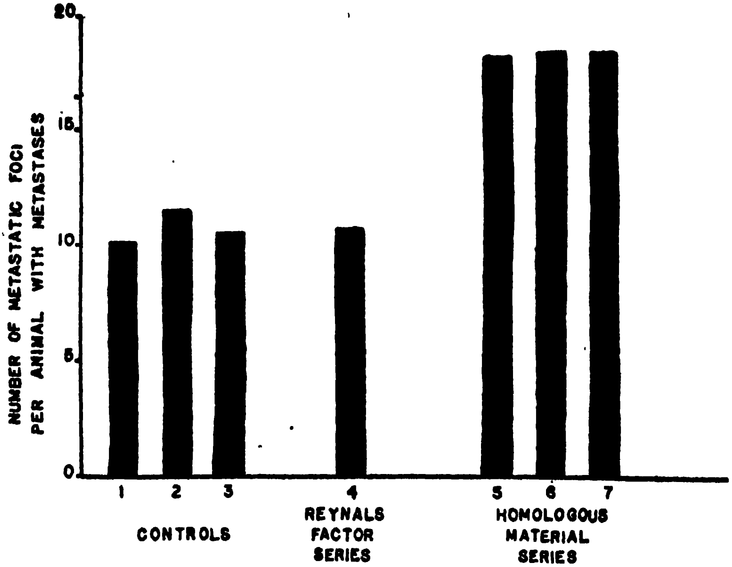

FIG. 1. Bio-assay of Duran-Reynals Factor and Homologous Material, with the Number of Metastatic Foci per Animal with Metastases as a Criterion

Columns 1 and 5 are based on an earlier paper (8); Columns 2, 4, and 6 on the present series of experiments; Column 3 represents an analysis of metastases among 367 miscellaneous control animals used in various experiments during a ten-year period; Column 7 represents the total experience to date with unfiltered and unaltered homologous tumor material (16 experiments, 94 rabbits).

longer or its effect on the tissues of the host is markedly prolonged. The finding that tumor-bearing animals show increased tumor growth following intravenous injection of testicle extract indicates that tests of homologous tumor materials should rely on a single injection of a small dose ten days or two weeks prior to tumor inoculation, and the determination of malignancy should depend not on local or primary growth but upon the number of metastatic foci or the mortality-longevity relationship. By this method confusion with the biological activity of testicle extract is avoided.

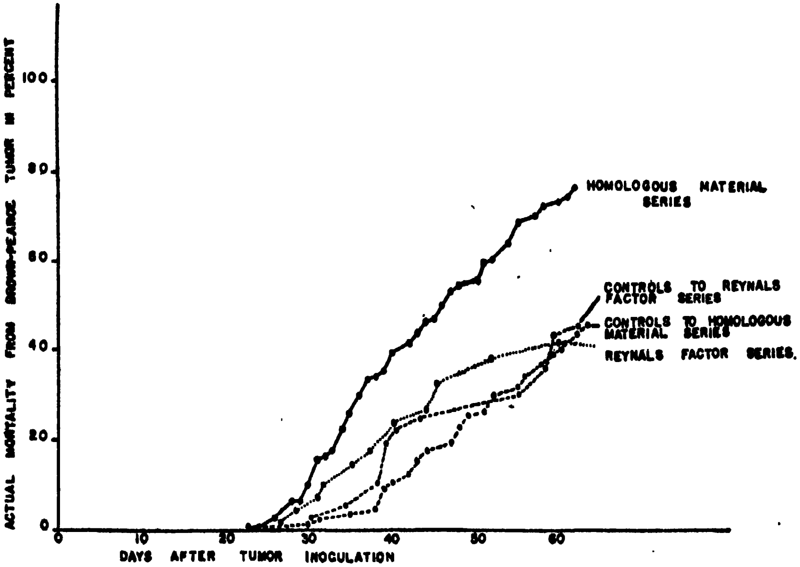

FIG. 2. Further Bio-assay of Duran-Reynals Factor and Homologous Material Based on the Mortality-Longevity Relationship

The homologous material series represents the total experience to date (16 experiments, 94 rabbits) and the controls in the same series (16 experiments, 98 rabbits); the other two curves are based on the present series of experiments.

SUMMARY AND CONCLUSIONS

Observations in a series of four experiments employing 76 rabbits and supported by a large collateral series indicate that an homologous material from the Brown-Pearce rabbit tumor described by the author is biologically different from the Duran-Reynals testicle extract factor. A single injection of 0.3 c.c. testicle extract two weeks before tumor inoculation failed to alter the incidence, volume, or distribution of primary or metastatic tumors, or to affect the mortality-longevity relationship, all of which values can be altered by homologous material similarly administered. A method of bio-assay of the homologous material is presented.

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VITAMIN C IN RELATION TO EXPERIMENTAL POLIOMYELITIS

With Incidental Observations on Certain Manifestations in Macacus rhesus Monkeys on a Scorbutic Diet

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Plates 30 and 31

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Data have recently been presented to show that the administration of natural vitamin C during the incubation period of experimental poliomyelitis is capable of modifying the course of the disease and of preventing paralysis in a considerable number of *Macacus rhesus* monkeys.

With the Aycock strain of the virus and the intracerebral route of inoculation, Jungeblut (1, 2) found that 77 of 243 (31.3 per cent) vitamin C-treated monkeys escaped paralysis, as compared with only seven of 136 (5.2 per cent) untreated animals. When synthetic vitamin C was used, no significant effect was obtained since only eleven of 101, or 10.2 per cent, failed to develop paralysis. With the M.V. virus (special strain used exclusively for nasal instillation for several years and obtained from the writer) instilled intranasally Jungeblut (2) reported that only one of twenty monkeys treated with 5 to 25 mg. of natural vitamin C daily (optimum dose in intracerebral experiments) showed no paralysis, while among ten animals treated with 50 to 100 mg. daily nine escaped paralysis; fifteen untreated monkeys all became paralyzed.

Since any substance exerting such an effect on experimental poliomyelitis might be a most useful therapeutic agent, it was deemed desirable to determine whether or not these results could be reproduced.

Methods

The strain of M.V. virus, the technique of nasal instillation, and other details which contribute to the constancy of infection (nearly 100 per cent in over 100 monkeys) by the nasal route were described in a recent communication (3).

The infecting dose was 1 cc. of a 5 per cent suspension of virus for each nostril on two occasions at an interval of 4 to 6 hours. When the second dose is omitted the number of monkeys which succumb may be reduced by half, indicating that the dosage employed is just within the range of that required to infect successfully all monkeys (provided the virus is fresh or has not been in glycerol much longer than a month). Two preparations of vitamin C, natural and synthetic,[1] were used. The vitamin was dissolved in distilled water just before using and the injections were given subcutaneously. When 400 mg. of the synthetic crystalline preparation was administered in a single daily dose, it was neutralized with NaOH just before using. The vitamin was administered immediately after nasal instillation of the virus and once daily thereafter until the outcome was clearly apparent.

Effect of Vitamin C on Disease Induced by Nasal Instillation of Virus

Thirty-six monkeys were used in the first test. They were on a vitamin C-adequate diet (oranges, bananas, bread, and milk) for at least 9 days before virus instillation, during which time their rectal temperatures were recorded daily as an aid in the interpretation of any subsequent febrile reaction. All monkeys were instilled at the same time with a pool of poliomyelitic cords which had been kept in 50 per cent glycerol for 3 months. Virus of this age was used with the hope that less than 100 per cent of the control animals would succumb. Ten monkeys were untreated; six received at the time virus was first instilled and daily thereafter 5 mg. of natural vitamin C; ten, 100 mg. of natural C; and ten, 100 mg. of synthetic, crystalline cevitamic acid.

The results recorded both in Chart 1 and Table I show that vitamin C neither modified the course of the disease nor prevented paralysis: 80 per cent of the untreated monkeys and 90 per cent of the treated ones developed paralysis. With these data at hand, Dr. Jungeblut's advice was sought and a similar experiment was carried out jointly in his laboratory. In a group of forty monkeys, among which ten were controls and thirty were treated with varying amounts of vitamin C, only one monkey, a treated one, escaped paralysis.

Influence of Preliminary Diet

In an attempt to find an explanation for the discrepancy between the present and the reported (1, 2) results, it appeared that vitamin C may perhaps be effective only at the time it is being taken up by depleted tissues. If that were the case, one might expect no effect in monkeys which had been in the laboratory on an adequate vitamin

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[1] Kindly supplied by Merck and Company.

C diet for some time prior to the experiment (as was the case in the present tests), while in monkeys whose store of vitamin C was depleted a beneficial result may perhaps be obtained. This possibility was submitted to experiment.

Forty-six monkeys, selected for their excellent physical condition and nutrition, were obtained in a single lot on Jan. 4, 1938, from one dealer who stated that they had arrived in a single shipment on Dec. 28, 1937, and that during the voyage, as well as in his own establishment, their diet included oranges and other fresh fruits. Twenty-one of these monkeys were placed on the regular laboratory diet (bread, pasteurized milk, oranges, and bananas) and at the end of 3 weeks only one died, the remaining twenty being healthy and well nourished. The other twenty-five monkeys were put on the following vitamin C-inadequate diet:

TABLE I

Effect of Vitamin C on Experimental Poliomyelitis

Series	Preliminary diet	Vitamin C given daily following nasal instillation of virus	Number of monkeys	Number developed paralysis
I	Vitamin C *adequate* for at least 9 days before test	None	10	8
		5 mg. natural	6 (1 died without evidence of poliomyelitis)	5
		100 " "	10	9
		100 " synthetic	10	9
II	Vitamin C *adequate* for at least 21 days before test	None	6	6
		100 mg. natural	7	7
		400 " synthetic	7	7
	Vitamin C *deficient* for 21 days before test	None	5	4
		100 mg. natural	7	7
Total untreated			21	18 (86 per cent)
Total treated			47	44 (94 per cent)

Milk (boiled with a constant stream of compressed air for 1 hr.)	250 cc.
Granulated sugar	15 gm.
Cod liver oil	15 cc.
Whole wheat bread	80 gm.
Peanuts	*ad lib.*

The indicated amounts were those allowed daily for each monkey. The diet was only partly consumed due to the monkeys' dislike for cod liver oil and when it was found that they were not thriving, it was changed on the 12th day to pasteurized whole milk with added cane sugar and 2 drops of drisdol (crystalline vitamin D in propylene glycol) per monkey, white bread, and peanuts; the new diet was completely consumed. At the end of 3 weeks thirteen of the twenty-five monkeys had died in a manner to be described in a subsequent section.

On Jan. 25, 1938, *i.e.*, at the end of 3 weeks on their respective diets, all the monkeys were given virus intranasally. Of the twenty which had been on the vitamin C-adequate diet, six served as controls, seven received 100 mg. of natural vitamin C daily, and seven others were given 400 mg. crystalline synthetic vitamin C daily at Dr. Jungeblut's suggestion, who believed from his unreported experiments that the synthetic vitamin might be effective when administered in larger amounts. Of the twelve remaining monkeys which were on the vitamin C-deficient diet, five served as controls and seven were given 100 mg. of natural vitamin C daily.

The results recorded in Table I and Chart 2 show no effect of vitamin C in either group. Of the thirty-two monkeys all but one developed paralysis and that one was in the untreated control group which had been fed with the vitamin C-deficient diet.

Some Manifestations in Monkeys on a Scorbutic Diet

Since hitherto few monkeys have been used in vitamin C studies, it may be of interest to record some of the more striking manifestations among the *rhesus* monkeys which were on a scorbutic diet in the present investigation. That which attracted most attention was the death of thirteen of twenty-five monkeys between the 9th and 18th days after they had been put on the scorbutic diet, while among the twenty-one monkeys on the regular diet all remained well during the same period.

Postmortem examination revealed evidence of acute infections in most of the fatal instances—five had non-tuberculous, lobar pneumonia with complete consolidation of one or more lobes; three, hemorrhagic enterocolitis; one, an erysipelas-like infection of the face; and in four there were no gross pathological changes other than emaciation; the monkeys that died with pneumonia were all well nourished (see Chart 3). While none of the monkeys showed any definite clinical or gross pathological evidence of scurvy, there was, nevertheless, microscopic evidence of early scurvy in the ribs and lower ends of the femurs of some of them.

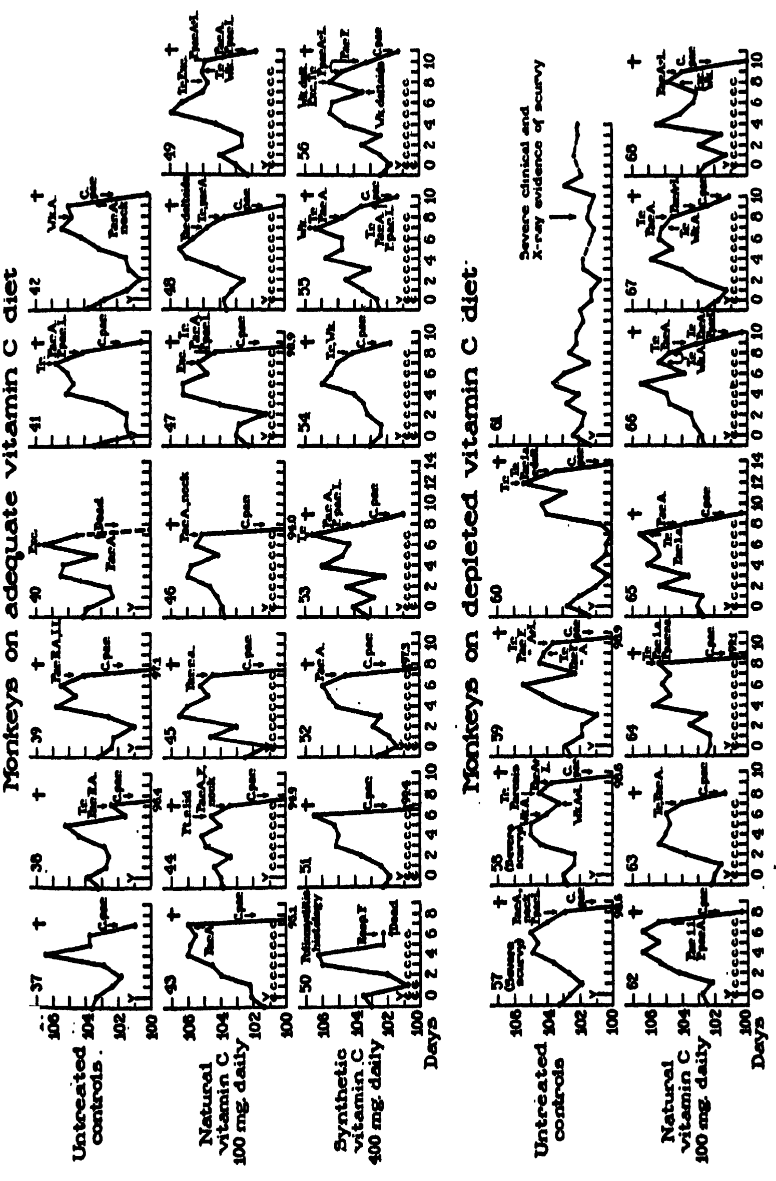

CHART 2. Influence of preliminary diet on subsequent effect of subcutaneous injections of vitamin C on the course of experimental poliomyelitis.

The increased susceptibility to spontaneous infection among guinea pigs on scorbutic diets and among human beings with scurvy has been noted by several investigators. It is also interesting that the infections observed by Höjer (4) and others in guinea pigs on vitamin C-deficient diets were chiefly of the upper respiratory tract and pneumonia, septicemia, and enterocolitis. The present observations on monkeys, which correspond so closely to those on guinea pigs, derive special significance from the practically total absence of acute infection among the other twenty-one monkeys of the same group which were receiving the regular diet.

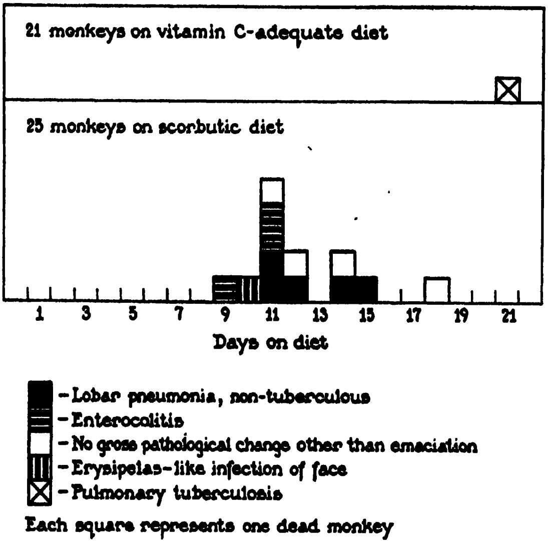

CHART 3. Mortality in relation to diet in a single colony of *rhesus* monkeys.

The twelve remaining monkeys all received poliomyelitis virus 3 weeks after the beginning of the vitamin C-deficient diet. Clinical, x-ray, or postmortem evidence of scurvy was found among three of the five monkeys whose diet was not supplemented by vitamin C, but not among the seven which received 100 mg. of natural vitamin C daily.

Two of these three showed their first signs of scurvy at the end of the 4th week after the beginning of the diet and during the course of poliomyelitic infection.

Monkey 58 first exhibited edema of the eyelids which became discolored the following day from spontaneous hemorrhages; there were also some sponginess and subgingival hemorrhage about three teeth. While there was no other external hemorrhage, the tourniquet test applied to the upper extremity yielded a large crop of petechiae on the arm and forearm distal to the tourniquet. There was a peculiar clumsiness of the upper and lower extremities (recorded as weakness) for at least 2 days before the onset of the characteristic nervous signs of poliomyelitis, apparently as a result of subepiphyseal fractures which were found at autopsy. The lower ends of both femurs and the upper ends of both tibiae and humeri showed varying degrees of fracture, periosteal separation, and subperiosteal hemorrhage (Figs. 1 and 2). "Mushrooming" of some of the ribs at the costochondral junctions could be discerned by palpation, as well as by x-ray, which also revealed "cupping," loss of calcium, and focal trabecular disintegration (Figs. 4 and 5). Outside the skeleton there was a striking reaction in the abdomen where there was considerable hemorrhage in the wall beneath the parietal peritoneum as well as large, subcapsular blood clots around both kidneys (Fig. 3). The writer has not found reference to hemorrhages in these sites and it is quite probable that they were the result of the method used in catching the monkeys, which involves grasping the animal by the small of the back. Monkey 57 showed gross changes only in the lower ends of both femurs (epiphyseal infractions) and by x-ray in some of the ribs. Monkeys 59 and 60 showed no gross scorbutic changes. Monkey 61 exhibited no evidence of poliomyelitic infection. On the 42nd day after the beginning of the diet, it had questionable gingival changes and also some difficulty in climbing; on the 43rd day both the upper and lower eyelids were edematous, and the gums were red and spongy about the incisor teeth. 2 days later the eyelids were hemorrhagic; it had lost the use of its right arm and could climb only with the greatest difficulty. The tourniquet test was positive on two occasions; it was negative in six normal monkeys. The monkey appeared ill, was disinclined to move, but it was quite evident that the loss of function in the extremities was due to spontaneous fractures—bony crepitus was clearly elicited—rather than to nervous involvement. X-ray revealed fractures with displacement at the lower ends of both femurs (Figs. 8 and 16); scorbutic changes were also apparent in the upper ends of the humeri and in the ulna at the wrists (Figs. 12 and 14). On completion of the 4 weeks of observation following the instillation of virus (*i.e.*, 49 days of deficient diet), 1 gm. of the crystalline, synthetic cevitamic acid used for treatment of the other monkeys was given subcutaneously to monkey 61. The following day the hemorrhage and edema of the eyelids had disappeared, the tourniquet test was negative, and the monkey appeared much improved. Smaller doses of vitamin C continued to be given daily and within 4 to 5 days it regained the use of its extremities and x-rays taken 10 days later showed repair of the fractures with calcification of what must have been extensive subperiosteal hemorrhages about the femurs, humeri, one ulna, and costochondral junctions (Figs. 7, 9, 11, 13, 15, and 17). These x-ray findings were confirmed at autopsy.

In correlating the manifestations in monkeys with the changes seen in human scurvy, it should be noted that the age of the monkeys used in these studies is equivalent to that of 10 to 13 years in human beings calculated on the basis of the development of ossification centers and the open epiphyses. The bony changes observed here correspond closely to those seen in scorbutic children. In so far as the high incidence of acute infections can be attributed to the inadequate diet, it is noteworthy that the increased susceptibility to spontaneous infection developed before any gross signs of scurvy had appeared. The impression was gained that in the investigation of certain problems relative to vitamin C deficiency *rhesus* monkeys might be used more advantageously than guinea pigs.

DISCUSSION AND SUMMARY

In the experiments reported in the present communication it was found that vitamin C, both natural and synthetic preparations, had no effect on the course of experimental poliomyelitis induced by nasal instillation of the virus. The objection cannot be raised that too large an amount of virus was used, since recent studies (3) on the fate of the nasally instilled virus indicated that all but an undetectable amount of it is swallowed and disappears from the nasal mucosa within 3 hours or less, and that none is demonstrable in the central nervous system before the 3rd day. Vitamin C administration was begun immediately after the instillation of virus and if it were capable of exerting any effect on the virus or the tissues it could have done so even before multiplication of virus had begun. Monkeys whose store of vitamin C was depleted reacted in the same way as those receiving an adequate diet. There is no apparent explanation for the difference between these results and those reported earlier by Jungeblut (1, 2).

During the present investigation it was found that monkeys on a scorbutic diet died of spontaneous acute infections, chiefly pneumonia and enterocolitis, while their mates receiving an adequate diet remained well. The surviving monkeys on the scorbutic diet developed the osseous and other changes of human scurvy, and the vitamin C used in this study was shown to produce healing and calcification in the bones as well as to check the edema and hemorrhagic diathesis.

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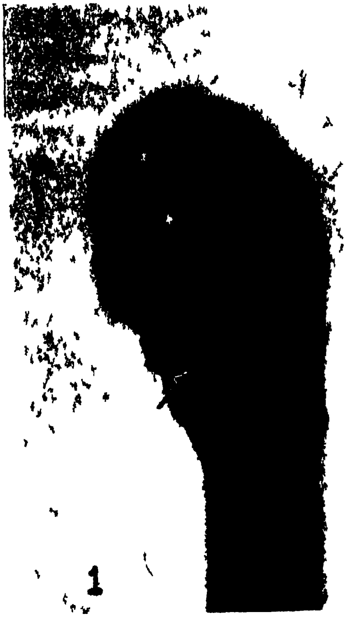
EXPLANATION OF PLATES

PLATE 30

FIG. 1. Lower end of femur of scorbutic monkey (M 58). Arrows point to subepiphyseal fracture and subperiosteal hemorrhage. × 2.

FIG. 2. Upper end of tibia of M 58. Note separation of periosteum from shaft without hemorrhage, in addition to subepiphyseal fracture. × 2.

FIG. 3. Subcapsular perirenal hemorrhage in M 58. × 2.



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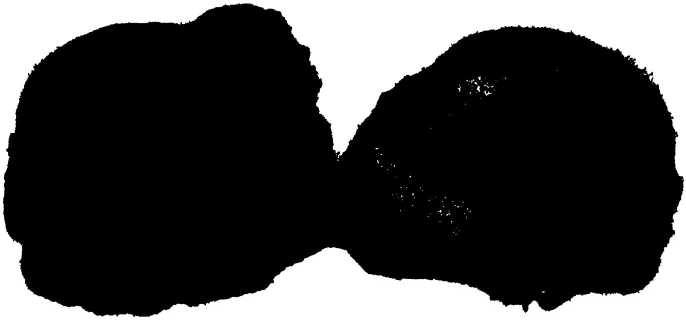

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Photographed by Joseph B Haulenbeek

(Sabin Vitamin C in relation to poliomyelitis)

PLATE 31

FIG. 4. Ribs of normal monkey. Note the almost straight line between the bone and cartilage. × 2.

FIGS. 5 and 6. Ribs of two scorbutic monkeys. Arrows point to "mushrooming" and "cupping" of costochondral junction and to foci of trabecular disintegration. × 2.

FIG. 7. Ribs of scorbutic monkey (M 61) 10 days after vitamin C. Note rosary formed by calcification of subperiosteal hemorrhage in all the fixed ribs with the exception of the upper three, suggesting the rôle of the respiratory movements in the causation of these changes. Actual size.

FIG. 8. Right knee of M 61 just before vitamin C therapy. Arrow points to fracture and displacement at lower end of femur; note also the light zone of diminished calcification.

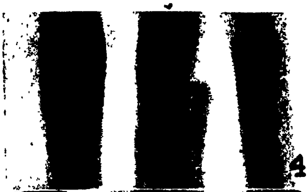
FIG. 9. Same knee 10 days after beginning of vitamin C therapy. Arrow points to calcified subperiosteal hemorrhage.

FIGS. 10 and 11. Right humerus (M 61) before and 10 days after vitamin C. Arrows in Fig. 11 point to calcified subperiosteal hemorrhage.

FIGS. 12 and 13. Right wrist (M 61) before and 10 days after vitamin C. Arrow points to the more marked zone of decalcification in the ulna prior to vitamin C.

FIGS. 14 and 15. Left wrist (M 61) before and 10 days after vitamin C. Arrow in Fig. 15 points to calcified subperiosteal hemorrhage.

FIGS. 16 and 17. Left knee (M 61) before and 10 days after vitamin C. Arrow in Fig. 16 points to fracture with displacement in femur and that in Fig. 17 to calcified subperiosteal hemorrhage.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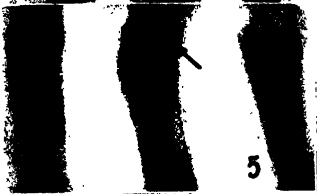
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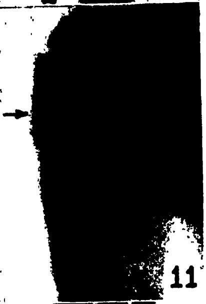
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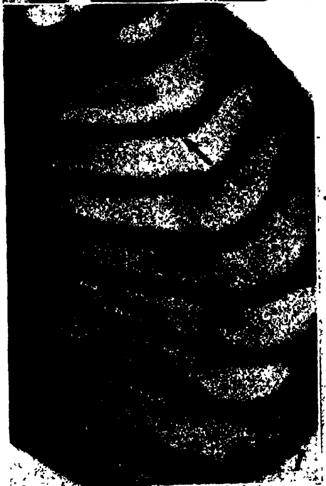
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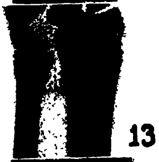
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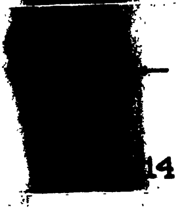
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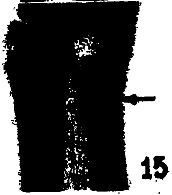
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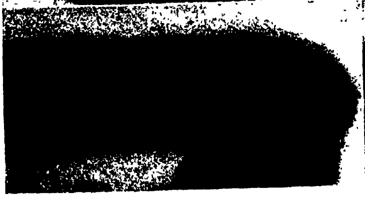

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(Sabin: Vitamin C in relation to poliomyelitis)

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EFFECT OF PREGNANCY UPON THE IMMUNITY OF MICE VACCINATED AGAINST ST. LOUIS ENCEPHALITIS VIRUS

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Swiss mice are uniformly susceptible to intranasal or intracerebral injection of St. Louis encephalitis virus. However, when the virus is injected subcutaneously it enters the general circulation but does not multiply in the brain. It produces no lesions in the nerve cells and causes no symptoms of encephalitis. However, such subcutaneous injection produces a strong immunity against subsequent intracerebral injection of the virus (1, 2).

The mechanism by which vaccination renders the brain cells immune is not known. During the course of investigations designed to study this matter, the question of the influence of pregnancy upon acquired immunity arose. Experiments were planned to determine whether pregnancy affected in any way the immunity induced by vaccination with the St. Louis virus.

Materials and Methods

Mice.—All mice employed were of the selected Swiss strain. They are uniformly susceptible to St. Louis encephalitis virus inoculated intracerebrally or intranasally. In all experiments the mated females and their virgin controls were of the same age.

Virus.—The virus used throughout was St. Louis encephalitis virus, strain 3. Virus suspensions were prepared in the following manner: The brain of a mouse prostrate with encephalitis was removed under sterile conditions and ground in a mortar. The emulsion of brain tissue was then diluted with ten times its weight of hormone broth of pH 8.0. After thorough mixing the suspension was centrifuged at 1,000 R.P.M. for 5 minutes and the supernatant made up in serial tenfold dilutions in broth.

Vaccination.—The mice were vaccinated with one subcutaneous injection of 0.5 cc. of a 1:1,000 dilution of virus in broth. On each occasion that the virus was used for vaccination it was titrated by intracerebral injection into virgin female Swiss mice. These titrations showed that the amount of virus used for vaccination was approximately 15,000 times the minimal lethal intracerebral dose. The subcutaneous injection of this quantity of virus never caused encephalitis.

Immunity Tests.—The immunity of the vaccinated mice was tested by intracerebral inoculation of the virus. All mice inoculated were under light ether anesthesia; each received 0.03 cc. of the virus appropriately diluted in broth. The immunity tests were carried out 2 weeks after vaccination in all experiments except Experiment 7. On each occasion that the virus was used to test immunity, it was titrated by intracerebral injection into unvaccinated virgin mice. These titrations showed that each vaccinated mouse tested for immunity received approximately 500 intracerebral M.L.D. of virus.

Mating.—Virgin females 2½ to 3 months old were chosen for mating. One male was mated to 5 females. As the females became pregnant, they were removed from the mating box to a separate jar. The date of delivery of the litter was recorded in each instance. Unless otherwise noted each litter was kept with its mother throughout the experiment.

Unmated Controls.—In each experiment a number of females were set aside as virgin controls. These animals were litter mates of those chosen for mating.

Determination of Pregnancy.—In most instances the pregnant mice delivered litters before the end of the experiment. The usual period of gestation of these mice was about 19 days. Mice failing to deliver litters 6 weeks after removal from the mating box were considered as non-pregnant. In one experiment some of the mated mice died a few days before the end of gestation. In these cases the presence of pregnancy was established by autopsy.

Autopsy Findings.—Practically all of the mice dying following intracerebral resistance tests developed typical symptoms of encephalitis. In the few doubtful cases the cause of death was proved to be encephalitis by histologic examination and by intracerebral inoculation of the suspected brains into mice.

EXPERIMENTAL

Susceptibility of Unvaccinated Virgin and Pregnant Mice to St. Louis Encephalitis Virus

Virgin mice are very susceptible to intranasally or intracerebrally inoculated St. Louis virus (1, 2). Numerous titrations of the virus in virgin Swiss mice 1 to 3 months old have shown that intracerebral inoculation of 0.03 cc. of a 1:1,000,000 dilution of virus is practically always fatal. The same quantity of a 1:10,000,000 dilution of virus kills about two-thirds of the mice injected. Table I shows that unvaccinated pregnant females exhibit the same degree of susceptibility to the virus as do unvaccinated virgin mice. It appears that the intracerebral minimal lethal dose of St. Louis virus is the same for the unvaccinated pregnant female as it is for the unvaccinated virgin female Swiss mouse.

Immunity Induced in Virgin Mice by Vaccination with St. Louis Virus

It has already been reported that within one week following vaccination the great majority of virgin Swiss mice become immune to 10,000 or more intracerebral minimal lethal doses of St. Louis encephalitis virus (1, 2). The immunity remains at this high level about 6 weeks, after which it begins to decline. Table II shows the degree of immunity exhibited by virgin female mice when tested 2 weeks after vaccination.

TABLE I

Susceptibility of Unvaccinated Pregnant and Unvaccinated Virgin Mice to St. Louis Encephalitis Virus

Mice	Dilution of virus injected intracerebrally (0.03 cc. of virus to each mouse)			
	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Unvaccinated virgin	4/4*	4/4	3/4	0/3
Unvaccinated pregnant†	3/3	3/3	2/3	0/3

* 4/4 = 4 of 4 injected mice died of encephalitis.

† These mice delivered litters 1 to 3 days after inoculation of the virus.

TABLE II

Immunity of Unmated Mice Induced by Vaccination with St. Louis Virus

Mice	Dilution of virus injected intracerebrally (0.03 cc. of virus to each mouse)						Immunity induced by vaccination
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
Vaccinated virgin	1/7*	0/8	1/8	0/8	–	–	10,000 intracerebral M.L.D.
Unvaccinated virgin (controls)	–	–	4/4	8/8	8/8	2/8	

* 1/7 = 1 of 7 mice injected died of encephalitis.

– = dilution not tested.

Influence of Pregnancy upon Immunity Induced by Vaccination

It appears from the data already presented that pregnancy does not render unvaccinated Swiss mice either more or less susceptible to the

St. Louis virus than are unvaccinated virgin mice. The experiments described below were planned to determine whether pregnant mice become immune following vaccination with the virus in the same manner as virgin mice.

Vaccination Late in Pregnancy.—

Experiment 1.—In this experiment the immunity induced by vaccination of mice during the last few days of pregnancy was compared with the immunity which follows vaccination of their unmated litter mates.

Twenty-eight female Swiss mice, 2½ months old, were divided into two groups, each consisting of 14 animals. Litter mates were equally distributed between the two groups. One group was mated with males of the same strain, while the other was set aside as unmated controls. All the mated females became pregnant and were not disturbed until 1 to 4 days before the end of the 19th day of the gestation period. At this time they were vaccinated subcutaneously with 0.5 cc. of a 1:1,000 dilution of St. Louis encephalitis virus. That is, the pregnant mice were vaccinated 15 to 19 days after conception. By titration of the virus used for vaccination it was estimated that the vaccinating dose employed was about 15,000 intracerebral M.L.D. Vaccination did not seem to interfere with the normal course of pregnancy; all the pregnant mice delivered normal litters 1 to 3 days after vaccination. The unmated mice were vaccinated at the same time as the pregnant animals, and with the same dose of virus. 2 weeks after vaccination the immunity of both parturient and unmated mice was tested by intracerebral inoculation of about 500 M.L.D. of St. Louis encephalitis virus.

A summary of this experiment is recorded in Table III. The mice vaccinated late in pregnancy failed to develop sufficient immunity to enable them to withstand 500 M.L.D. of the virus. 13 of 14 of them died of encephalitis following the test inoculation of virus, a mortality rate of 93 per cent. In contrast, only 1 of the 14 vaccinated unmated mice died after the intracerebral administration of the virus, a mortality rate of 7 per cent. Calculation of the standard error (3) indicates that this difference in mortality is probably not an accidental one. The result obtained might be expected to occur by chance much less frequently than one time in 100 ($P = < 0.01$).

When the offspring of the parturient vaccinated females became 10 to 13 days old, their immunity against intranasal instillation of St. Louis virus was tested. 92 of 98 of the infant mice inoculated with 100 intranasal M.L.D. of virus died of encephalitis.

Experiment 1 a.—This experiment was carried out in exactly the same manner as Experiment 1. The result was practically identical. 12 of 13 mice vaccinated 1 to 4 days before the end of pregnancy died of encephalitis after the intracerebral immunity test, a mortality rate of 92 per cent.

As before, the great majority of the vaccinated unmated mice were immune; only 2 of 15 (13 per cent) failed to withstand the test dose of virus. Again $P = < 0.01$.

TABLE III

Effect of Pregnancy upon Immunity When Vaccinating Dose Is Administered near End of Pregnancy

Immunity of pregnant mice* following vaccination			Immunity of unmated mice following vaccination	
Vaccinated pregnant mouse No.	Time of vaccination Day of pregnancy	Fate after intracerebral test for immunity (500 M.L.D. of virus)	Vaccinated unmated mouse No.	Fate after intracerebral test for immunity (500 M.L.D. of virus)
1	18th	Died of encephalitis	1	Remained well
2	17th	" " "	2	" "
3	17th	" " "	3	" "
4	16th	" " "	4	" "
5	18th	" " "	5	" "
6	18th	Remained well	6	" "
7	16th	Died of encephalitis	7	" "
8	18th	" " "	8	" "
9	17th	" " "	9	" "
10	16th	" " "	10	" "
11	16th	" " "	11	" "
12	16th	" " "	12	Died of encephalitis
13	16th	" " "	13	Remained well
14	16th	" " "	14	" "

Mortality of vaccinated pregnant mice = 93 per cent (13/14); mortality of vaccinated unmated mice = 7 per cent (1/14).

* Duration of pregnancy is 19 days in these mice.

The offspring of these vaccinated mothers also showed little evidence of immunity. Of the 80 young mice tested 75 died of encephalitis after receiving 100 intranasal M.L.D. of virus.

Vaccination during Middle Third of Pregnancy.—

The effect of vaccinating pregnant mice during the middle third of the gestation period was next studied as described in Experiment 2.

The result indicated that the immunity induced by vaccination of mice at this stage of pregnancy was also diminished.

Experiment 2.—This experiment was conducted in the same manner as those already described except that the pregnant mice were vaccinated earlier in the gestation period, that is, 7 to 11 days after the onset of their pregnancy. 2 weeks after vaccination the immunity of the unmated and of the parturient mice was tested by intracerebral inoculation of approximately 500 M.L.D. of virus.

Following this test dose of virus, 9 of 18 vaccinated pregnant mice died of encephalitis, a mortality rate of 50 per cent. As before, the vaccinated unmated mice proved to be immune, only 1 of 15 (7 per cent) died of encephalitis ($P = < 0.01$).

Vaccination Early in Pregnancy.—

Experiment 3.—In this experiment 22 mice were vaccinated during the first 4 days of pregnancy. Their immunity, together with that of vaccinated unmated controls, was tested 2 weeks after vaccination.

The response to vaccination was found to be depressed even very early in the gestation period. 11 of the 22 vaccinated pregnant mice (50 per cent) died of encephalitis after intracerebral inoculation of approximately 500 M.L.D. of virus as compared with only 1 of 14 (7 per cent) of the vaccinated unmated mice ($P = < 0.01$).

Vaccination during the Puerperium.—

The experiments described above indicated that pregnancy inhibits or depresses the immunity which is produced by vaccination. It seemed important to learn whether the power to acquire immunity against the virus was regained after the mice had delivered their young. Accordingly, the response to vaccination carried out at various intervals after the end of pregnancy was studied in Experiments 4, 5, and 6 described below. It was found that the degree of immunity exhibited by the vaccinated puerperal mice during the first 2 weeks after delivery of their young was less than that of vaccinated virgin mice. However, mice vaccinated 7 weeks after the end of pregnancy appeared to withstand the intracerebral inoculation of 500 M.L.D. of the virus as well as did vaccinated virgin mice. Apparently at this time the mice which have reared young have regained at least in part their ability to acquire immunity against the virus. Text-fig. 1 summarizes these results.

Vaccination during the First 2 Days after the End of Pregnancy.—

Experiment 4.—13 Swiss mice were vaccinated against St. Louis virus within the first 48 hours after they had delivered litters. At the same time 12 virgin females were vaccinated. 2 weeks later the immunity of all the mice was tested in the usual way against intracerebrally inoculated St. Louis encephalitis virus.

Seven of the 13 vaccinated puerperal mice (54 per cent) died of encephalitis while only 1 of the 12 vaccinated virgin mice (8 per cent) failed to survive the test virus ($P = < 0.01$).

Vaccination during the 2nd Week after the End of Pregnancy.—

Experiment 5.—This experiment was performed in the same manner as Experiment 4, except that the mice which had borne litters were vaccinated later in the puerperal period; that is, 8 to 12 days after delivery of their young.

Seven of 15 (47 per cent) of the mice vaccinated during the puerperium died of encephalitis following the intracerebral test inoculation of 500 M.L.D. of virus. None of 14 vaccinated unmated mice failed to survive the immunity test ($P = < 0.01$).

Vaccination 7 Weeks after the End of Pregnancy.—

Experiment 6.—In this experiment the mice were vaccinated 7 weeks after they had delivered their litters; that is, 4 weeks after weaning of their young.

Twenty female Swiss mice were mated with 4 males, while 14 of their litter mates were kept as virgin controls. All the mated females delivered litters 19 to 23 days after mating. 50 to 54 days after delivery the young were weaned and removed from their mothers and discarded. About 4 weeks later (more than 7 weeks after delivery) the mice which had reared young and the virgin controls were vaccinated against St. Louis virus in the usual way. 2 weeks later the vaccinated mice were inoculated intracerebrally with about 500 M.L.D. of St. Louis encephalitis virus.

None of the 14 vaccinated virgin controls died of encephalitis and only 2 of the 20 vaccinated mothers (10 per cent). This difference is probably due to chance ($P = 0.21$).

Effect of Pregnancy upon Immunity Established by Vaccination before Mating.—

It seems clear from the data presented that mice vaccinated during pregnancy are in some manner prevented from developing strong immunity against the St. Louis virus. The effect of pregnancy upon immunity already well established before the onset of gestation was investigated in Experiment 7. The result of Experiment 7 (summarized in Table IV) shows that pregnancy brings about a reduction of the immunity acquired before mating.

Experiment 7.—46 female Swiss mice were vaccinated with St. Louis virus in the manner previously described. 1 week later 11 of them were chosen at random and their immunity tested against approximately 500 intracerebral M.L.D. of virus. All of these mice survived the immunity test and it was therefore assumed that the remaining 35 vaccinated mice were also immune. 1 week after vaccination 19 of these latter animals were mated to 5 males of the same strain while 16 were set aside as virgin controls. 16 days later (23 days after vaccination) the males were removed and the immunity of the mated and virgin vaccinated females was tested by intracerebral inoculation of approximately 500 M.L.D. of virus. Of the 19 mated mice, 18 either delivered litters before the end of the experiment or were found by autopsy to be within 3 or 4 days of term when death from encephalitis occurred.

TABLE IV

Effect of Pregnancy on the Immunity Established by Vaccination before Mating

Mice	Time mated	Time of intracerebral immunity test	Mortality following intracerebral immunity test (500 M.L.D. of virus)
Vaccinated virgin........	—	1 wk. after vaccination	0/11* (0 per cent)
Vaccinated virgin.........	—	23 days after vaccination	2/16 (13 per cent)
Vaccinated, then mated (pregnant).............	1 wk. after vaccination	23 days after vaccination (16 days after mating)	12/18† (67 per cent)

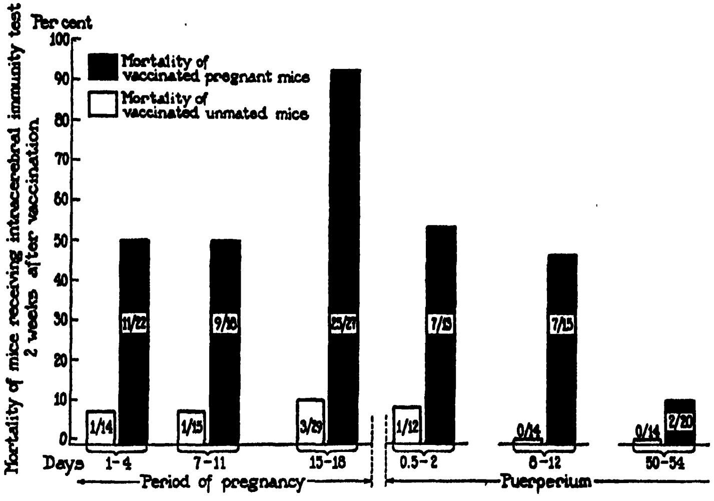
* 0/11 = none of 11 injected mice died of encephalitis.

† Of the 6 survivors in the pregnant group, 3 had been pregnant only 1 or 2 days when given the test virus.

Following the inoculation of the test virus, 12 of the 18 pregnant mice (67 per cent) died of encephalitis as contrasted with only 2 of 16 (13 per cent) of the vaccinated unmated animals ($P = < 0.01$). It is of interest to note that of the 6 vaccinated pregnant mice which survived 3 had been pregnant only 1 or 2 days and 2 only 10 days when the immunity test was done. All the vaccinated pregnant mice which died of encephalitis had been pregnant 14 to 16 days when they received the test virus.

DISCUSSION

There are certain indications in the literature that various physiologic factors may influence the reaction of animals to virus infections. For example, it seems clear that the age of the animal may be important in this regard. It has been shown by Sabin and Olitsky (4) that old mice are immune to exceedingly large doses of vesicular stomatitis virus injected into the leg muscles, while young mice develop a fatal encephalomyelitis. Sabin stated that in young mice the virus multiplies at the site of inoculation and invades the sciatic nerve and spinal cord, while in old mice this invasion of the central nervous system does not take place.



TEXT-FIG. 1. Effect of pregnancy upon immunity produced by vaccination against St. Louis encephalitis virus.

It has been reported by Sprunt (5) that pregnancy alters the reaction of the tissues of the rabbit to the virus of infectious myxomatosis. Sprunt reported that following intradermal inoculation of this virus more extensive lesions occur in the spleens of pregnant animals than in unmated ones, and that secondary lesions are found uniformly in the lungs of pregnant animals, while only an occasional lesion is found in the lungs of non-pregnant rabbits. On the other hand the skin of the pregnant animals seems to be less affected by the virus than is the case in the non-pregnant rabbits.

The experiments described in the present communication show that pregnancy does not increase the susceptibility of mice to the St. Louis encephalitis virus, but pregnancy does very definitely diminish the response to vaccination with the virus. This depression of the ability to acquire immunity is most marked late in pregnancy, but it is also demonstrable very early in pregnancy as well as during the first 2 weeks postpartum. However, the immunity response is not permanently reduced by pregnancy. When mice are vaccinated 7 weeks after the end of pregnancy, it is found that their ability to acquire immunity has been regained, at least in part.

It is of interest to note that the offspring of the mice vaccinated during the course of pregnancy are not immune to the virus. Very few of these young mice were able to withstand the administration of 100 intranasal M.L.D. of the virus.

It should be emphasized that pregnancy not only inhibits the development of immunity but that it also adversely affects an already well established immunity. This would suggest that pregnancy exerts its influence not by altering the virus injected as the vaccinating agent but rather that it affects in some manner the tissues which are made immune by vaccination. By what mechanism the state of pregnancy brings about this alteration is not known. Pregnancy is accompanied by very profound physiologic changes in the animal. It seems likely that one or more of these metabolic changes are responsible, although it is impossible at the present time to say which are involved. Experiments designed to study some of these factors, such as the hormones which exert a great influence on the course of pregnancy, are being undertaken.

SUMMARY

1. Virgin and pregnant Swiss mice are equally susceptible to intracerebral inoculation of St. Louis encephalitis virus.

2. Following subcutaneous vaccination with the St. Louis virus, the great majority of virgin Swiss mice become immune to subsequent intracerebral injection of 10,000 M.L.D. of the virus.

3. The majority of mice vaccinated during pregnancy do not become immune to even as little as 500 intracerebral M.L.D. of the virus. The depression of the ability to acquire immunity against the virus is most marked when the vaccination is carried out late in pregnancy, but it is also demonstrable when the mice are vaccinated early in the gestation period and during the first 2 weeks postpartum. At 7 weeks postpartum the response to vaccination is more nearly like that of virgin mice.

4. Pregnancy not only interferes with the development of acquired immunity but it also diminishes a previously established immunity.

5. Offspring of the mice vaccinated during pregnancy are not immune to 100 M.L.D. of virus.

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THE RELATION BETWEEN CONDUCTION VELOCITY AND THE ELECTRICAL RESISTANCE OUTSIDE A NERVE FIBRE

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Nervous transmission is generally believed to depend upon an electrical process, but the precise way in which one section of a nerve fibre activates another is still uncertain. According to the membrane theory, restimulation is brought about by the local electric currents which spread in advance of the active region. In the conventional form of the theory the current is assumed to flow in one direction along the core of the fibre and to return through the conducting fluid outside. If this view is correct, the velocity of transmission should vary with the electrical resistance outside a nerve fibre. Thus in Lillie's iron wire model, the velocity of propagation may be altered over a wide range by changing the volume and hence the total conductance of the fluid outside the wire [Lillie, 1924]. The experiments to be described in this paper were undertaken in order to decide whether the rate of propagation in a nerve fibre can be affected in a similar way. Single fibre preparations from crab or squid were employed, and the external resistance was changed by lifting the fibres out of sea water into a layer of aerated paraffin oil, or in the case of the squid fibres into an atmosphere of moist air. In sea water the fibres are shunted by a large volume of conducting fluid, but in oil or air they are surrounded by a thin film of saline, so that the external resistance would be high. This method of changing the external resistance has the advantage that the fibres are always in contact with a layer of sea water, so that their immediate environment remains unchanged throughout the experiment. Hence any changes in velocity produced by this treatment must be entirely due to the alteration in electrical resistance and cannot be attributed to chemical or ionic effects.

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Experiments with Crab Axons

Single fibres were isolated from the limb nerve of *Carcinus maenas* by a method which was described in a previous paper [Hodgkin, 1938]. An account of the stimulating and recording apparatus was also given in this paper, so that they need not be described here. The arrangement for comparing conduction velocity in oil and in sea water is shown in Fig. 1. It consisted of four platinum electrodes (*B*—*E*), a fine glass hook (*G*) and two holders (*A*, *F*) which were made from fine-tipped forceps. When the dissection was complete, the electrode system was lowered into the sea water and one end of the fibre was gripped by the holder *A*. The other end was then seized with a needle and the intermediate stretch looped above the electrodes and beneath the glass hook. Finally, the free end of the fibre was gripped in the second holder *F*, and the glass hook was gently lowered until the fibre was taut. The whole system of holders and electrodes was firmly attached to a movable stage which could be raised or lowered by means of the screw adjustment on a Palmer stand. In order to measure the conduction velocity in sea water, the fibre was raised until the electrodes *C* and *D* were just clear of the interface. At this point both stimulating and recording leads were insulated by oil, but the greater part of the intermediate conduction distance was immersed in sea water. In order to determine the conduction velocity in oil it was only necessary to raise the electrode system until the whole of the fibre had passed through the interface.

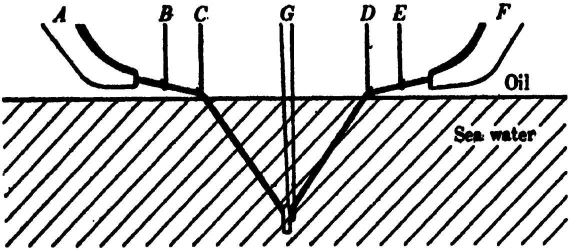

FIG. 1

The results of a typical experiment are illustrated by Fig. 2. In *A* the intermediate conduction stretch was immersed in sea water and in *B* it was raised into oil. This had a marked effect on the conduction rate, since the velocity was about 30% greater in *A* than in *B*. The change was completely reversible, for the conduction rate returned to its original value when the fibre was replaced in sea water (*C*).

In order to make certain that the observed changes were not due to alterations in latency, the leads were arranged so that the action potential could be recorded before it entered the sea water as well as after. Measurements of this kind gave an entirely satisfactory result, for they showed that the latency did not change by more than 50 μsec., whereas the total conduction time might alter by as much as 1000 μsec.

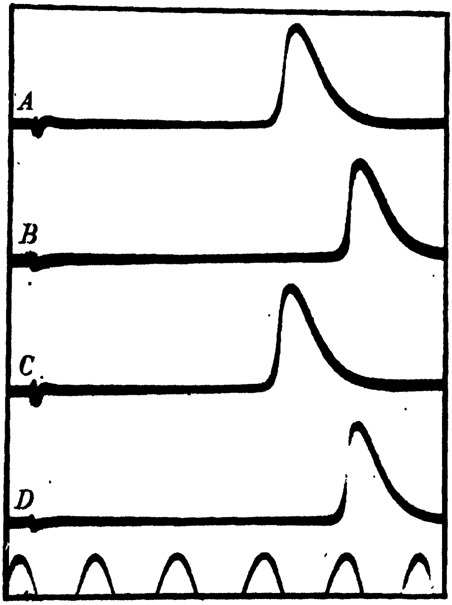

FIG. 2. *A* and *C*, action potential recorded with sea water covering 95% of intermediate conduction distance, *B* and *D*; fibre completely immersed in oil. Conduction distance 13 mm. Time msec.

By lowering different fractions of the nerve fibre into sea water it was possible to show that the increase in conduction rate must occur to a uniform extent throughout the fibre. Fig. 3 illustrates the result of an experiment of this kind. In *A* the entire conduction distance was surrounded by oil, and in *B* 95% of it was covered by sea water. In *C* one-third was raised into oil and two-thirds were left in sea water, whereas in *D* two-thirds were in oil and only the central third remained in sea water. Finally, in *E*, the entire conduction distance was again raised into oil. This experiment shows that the decrease in conduction time is roughly proportional to the length of nerve immersed in sea water, as it should be, if the effect depends upon a uniform increase in conduction velocity.

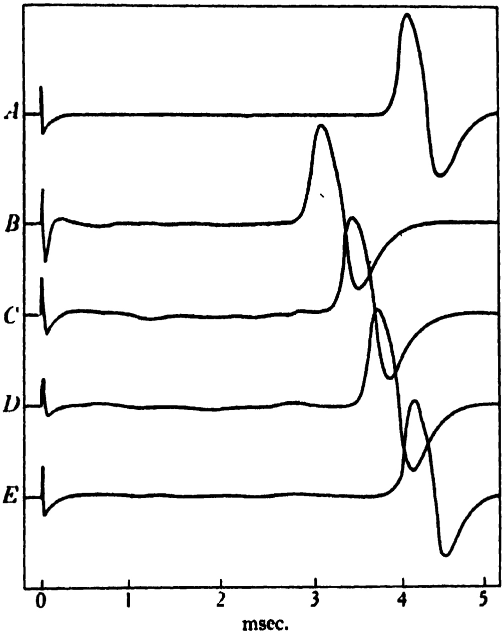

FIG. 3. *A*, *E*, fibre completely immersed in oil. *B*, 95% of conduction stretch in sea water. *C*, 67% in sea water. *D*, 33% in sea water. Conduction distance 13 mm.

In some of the records in Fig. 3 the base-line is disturbed by small irregularities which occur between the application of the stimulus and the arrival of the action potential. These disturbances usually occur at the moment when the action potential crosses the oil-sea water interface or passes some other discontinuity on the nerve. They are to be regarded as artifacts and occur because the potential

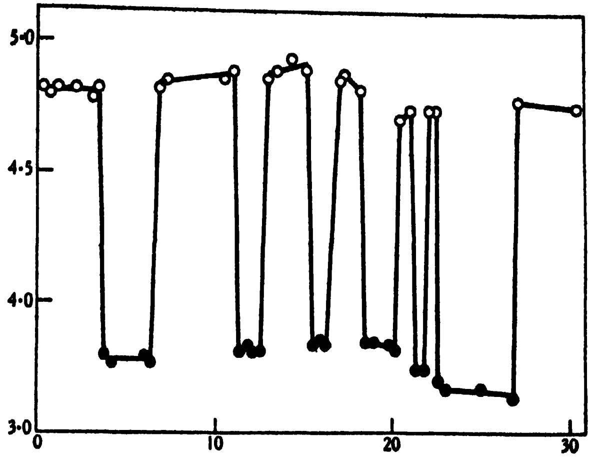

FIG. 4. Abscissa: time in minutes. Ordinate: conduction velocity in m./sec. Hollow circles: velocity in sea water. Full circles: velocity in oil.

TABLE I

Conduction velocities of crab fibres in oil and sea water. The diameter of the axis cylinder of these fibres was about 30 μ. Temperature about 21°C. In certain cases the conduction distance was not measured, so that the absolute values for velocity were unknown, although the percentage increase could be obtained from the conduction times.

Velocity in oil m./sec.	Velocity in sea water m /sec.	Percentage increase
—	—	35
—	—	33
2.8	3.9	40
4.1	5.3	30
3.8	4.9	28
—	—	33
3.4	4.5	33
—	—	25
3.6	4.1	14
3.4	4.0	18
3.5	4.0	15
3.0	3.9	30
2.6	3.1	20
4.4	5.5	26
4.0	5.2	30
3.5	4.5	29

difference between the ends of the nerve fibre undergoes a sudden change at the moment when the action potential enters or leaves the sea water, thus producing a transient flow of current through the stray capacities between the stimulating and recording leads. Artifacts of this kind were not observed in similar experiments with the squid giant fibre, because this has a much lower resistance, so that effects due to stray capacity are relatively unimportant.

The process of transferring a fibre from oil to sea water occupied only a few seconds, so that a large number of observations could be made on one fibre. A series of measurements extending over a period of 30 min. is shown in Fig. 4, and provides a good demonstration of the speed and reversibility of the velocity change.

Table I summarizes the results of the experiments with crab fibres and shows that there is considerable variation in the magnitude of the velocity change. This probably depends upon differences in the amount of connective tissue left on the fibres, since any adherent tissue must lessen the external resistance in oil and so facilitate the propagation of the action potential.

Experiments with the Squid Giant Axon

The giant fibres used in this work were obtained from the hindmost stellar nerve of *Loligo pealii* [Young, 1938] by the method described by Cole & Curtis [1939].

The stimulating and recording apparatus were slightly different from those used in the previous experiments, but need no special description. The electrode system was similar in principle to that in Fig. 1, except that the leads were sealed into a wax cell and the axon was immersed in sea water by raising the interface instead of by lowering the electrodes. The giant fibre survived quite well in an atmosphere of moist air, so that it was unnecessary to use oil as an insulating medium and the external resistance could be changed by lowering the sea water until the whole fibre was surrounded by air.

The results of a typical experiment are shown in Fig. 5. Record *B* was made with the conduction distance in moist air, and *A* and *C* with the sea water covering about 95% of its length. In this experiment there was no visible shock artifact, but this was of little consequence, since the stimulus was arranged to coincide exactly with the beginning of the sweep, so that the conduction time could be obtained from the interval between the start of the sweep and the arrival of the action potential. Thus a comparison of records *A* and *C* with the time base shows that the conduction time was 1.15 msec.[1] In *B* it

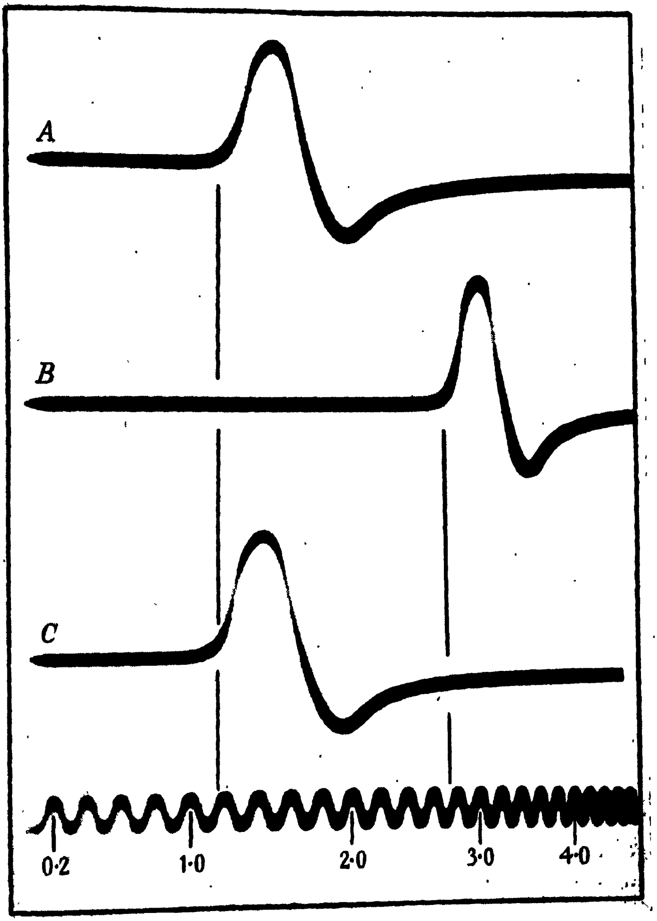

FIG. 5. *A*, *C*, velocity with 95% of conduction distance in sea water. *B*, whole fibre in moist air. Time base 5000 cycles. The figures give the time in msec. after the shock. Note that the sweep is exponential. Conduction distance 26 mm.

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[1] Conduction times were measured between the beginning of the shock and the moment when the action potential rose to 5% of its maximum.

was 2.75 msec., so that the velocity must have been about 140% greater in sea water than in air. This increase was the largest observed in the present work, but it was by no means abnormal, since changes of 100% or more have been observed on several occasions. Table II summarizes the results of the experiments on squid axons.

These large changes could only be obtained when the axons were very carefully cleaned from all loose connective tissue. A good illustration of this is afforded by an experiment in which measurements were made before the final cleaning of the fibre had been completed. Under these conditions the following values were obtained:

TABLE II

Conduction velocities of squid fibres in air and sea water. The axis cylinders of these fibers were between 480 and 520 μ in diameter. Temperature, about 20°C. The low velocities in the second experiment probably occurred because this fibre was kept for 11 hr. before being used. One preliminary experiment in which a 60% increase was obtained has not been included.

Velocity in air m./sec.	Velocity in sea water m./sec.	Percentage increase
11.7	25.0	114
(5.6)	(10.6)	89
9.5	22.6	138
13.8	26.8	97
11.3	25.5	134

Velocity in sea water = 25.4 m./sec.
Velocity in air = 17.7 m./sec.
Increase = 44%.

The fibre was then replaced in sea water for 3 hr. and as much connective tissue as possible removed from it. When this operation was complete the velocities were again measured and found to be as follows:

Velocity in sea water = 25.0 m./sec.
Velocity in air = 11.7 m./sec.
Increase = 114%.

This experiment shows that the removal of connective tissue had no appreciable effect on the velocity in sea water, and indeed there is no reason why it should, since the resistance of a large volume of sea water is so low that the velocity would be limited only by the internal resistance of the axis cylinder. However, in air the adherent tissue plays an important part, since it lowers the external resistance and thus allows the action potential to propagate faster than it would in a cleanly dissected fibre. Similar considerations also account for the quantitative difference between crab and squid fibres, since the ratio of adherent material to axis cylinder is greater in crab fibres, so that the velocity change should be smaller than in the squid preparation.

Velocity in Nerve Trunk

Experiments with whole nerve trunks showed that the velocity of the giant axon *in situ* was approximately equal to the velocity of the same axon in a large volume of sea water. This result seems reasonable, since the resistance of the nerve trunk would be low compared with that of the axis cylinder, so that the electrical conditions would approach those of an isolated axon in sea water.

Similarity in the Action of Oil and Air

In most of the work on squid axons air was used as an insulating medium, but on two occasions oil was used, in order that its action might be compared with that of air. These experiments showed that both media produced approximately the same decrease in velocity, which is a satisfactory result since it indicates that the effect of paraffin oil is solely due to its properties as an insulator.

Alteration of Conduction Velocity by Metallic Conductors

A few experiments were made in order to discover whether the conduction velocity could be accelerated by means of a metallic short circuit. This was done by placing a grid of platinum strips between the stimulating and recording leads. These were sealed into the moist chamber and arranged so that they could be connected together by means of a mercury switch (Fig. 6). The fibre was mounted in position, and the conduction velocity measured with the switch first in one position and then in the other. This method of changing the external resistance has the advantage that the fibre itself remains in a constant environment and the only condition altered is the position of a switch outside the moist chamber. Measurements were made on three axons and in each case the conduction velocity was found to be greater when the metal strips were connected together (Table III). The effect was not large, but it was striking to watch on the oscillograph screen, since it took place within the fraction of a second required to move the switch. Table III shows that the change in velocity was much smaller than that observed in the air-sea water experiments. There are two good reasons for this difference. In the first place, the metal bridges are polarizable, so that they offer an appreciable resistance to the action potential; and in the second place they only connect certain discrete points on one side of the fibre, instead of short-circuiting the entire surface as sea water does.

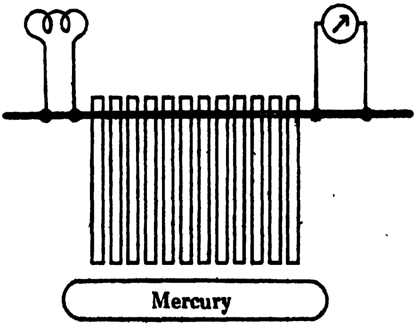

FIG. 6. Diagram of apparatus for short-circuiting giant fibre with metal conductors.

TABLE III

The velocities given are the average velocities between stimulating and recording leads. In the first case, the conduction distance was not measured.

Velocity with strips disconnected	—	14.7 m./sec.	12.5 m./sec.
Velocity with strips connected	—	17.8 m./sec.	14.3 m./sec.
Percentage increase	12	21	14

Metal Bridge Experiment

One preparation afforded an opportunity for observing a phenomenon which resembled that in the *Nitella* salt bridge experiment [Osterhout & Hill, 1930]. A fibre was left in the electrode chamber at the end of an experiment, and after a little time it was found that a spontaneous block had developed between two of the platinum strips. This was only effective when the strips were disconnected; if they were joined by the mercury contact, the action potential was able to traverse the injured region and so reach the recording leads. This condition did not last long, because the intensity of the block soon deepened to a point where it could no longer be relieved with the short circuit. There is no reason to doubt this effect in spite of its transient nature, for the result is an obvious corollary to the preceding experiments.

DISCUSSION

The results of the present research are very similar to those obtained by Auger [1933, 1936]. Auger measured the velocity of propagation in *Nitella*, and showed that it could be accelerated by covering the *Nitella* cell with a strip of moist filter paper. He considered that the increase in velocity could only be due to the decrease in external resistance, and concluded that his results constituted strong evidence for the local circuit theory. With the exception of this experiment on *Nitella*, all previous attempts to demonstrate a relation between conduction rate and electrical conductance have depended upon altering the chemical composition of the fluid bathing the tissue. Thus Pond [1921] immersed skeletal and cardiac muscle in solutions of low salt content and showed that this treatment led to a reduction in conduction velocity. This experiment is suggestive, but is not completely conclusive, because the observed change in velocity might have been due to an alteration in membrane excitability resulting from the decrease in salt content. This objection can be raised to any method which depends upon altering the constitution of the external medium. It does not apply to the present experiments, because in them the external resistance was changed by altering the volume and not the chemical constitution of the fluid outside the nerve fibre.

SUMMARY

The following experiments show that the speed of propagation depends upon the electrical resistance outside a nerve fibre:

1. The conduction rate of an isolated crab fibre was 14–40% faster in sea water than in oil. The external resistance was increased

by oil, because the fibre was surrounded by only a very thin film of salt solution.

2. The velocity in the giant axons of the squid was 80–140% faster in sea water than it was in air or oil.

3. A giant axon was laid on a series of metal strips which could be joined by a mercury contact. The velocity was accelerated by 12–21% when the strips were connected together.

These results strengthen the local circuit theory of nervous transmission.

The work on squid axons was made possible by the kindness of Dr Cole and Dr Curtis, who demonstrated the dissection of the giant fibre and allowed me to use their equipment at Woods Hole. I wish to express my gratitude to them, and at the same time to Dr Gasser for assistance and hospitality in New York.

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THE KINETICS OF PENETRATION

XVI. THE ACCUMULATION OF AMMONIA IN LIGHT AND DARKNESS

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(Accepted for publication, December 30, 1938)

In a former paper[1] the accumulation of ammonia[2] by *Valonia macrophysa*, Kütz., was discussed. In the present paper we compare the rates, and steady states, in cells exposed to sea water containing ammonia in light[3] (regular succession of daylight and darkness) and in the dark. At the same time the pH changes have been examined by an improved method which avoids the loss of gas from the sap.

The experiments were carried out in Bermuda in the winter of 1937–38 at the Bermuda Biological Station for Research.

EXPERIMENTAL

In these experiments the cells were exposed in large jars to the sea water containing ammonia. In the dark experiments the glass jars were made dark by layers of adhesive tape covered with a thick layer of either black paint or a very opaque aluminum paint. In some cases, however, stoneware jars were used. In all cases the jars were stoppered and were immersed up to their necks in flowing salt water from the Biological Station salt water system. The temperature remained fairly constant at 17°C. ± 1°C. during the course of the experiments.

Analyses for potassium and sodium were each carried out on 0.1 ml. samples as described in previous papers[1] except that in place of the Kuhlmann micro balance, a Becker semi-micro balance was used. Weighings were made to 0.02 mg. which in most cases represented a weighing error of less than 0.5 per cent. This accuracy was quite sufficient in view of the variability of the material.

The ammonia analyses were carried out by means of Nessler's reagent using the method described in the previous paper. The quantity of sample varied from

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[1] Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1930–31, **14**, 301.

[2] To prevent misunderstanding we shall define ammonia as $NH_3 + NH_4^+ + NH_4OH$; *i.e.*, the total amount found by analysis. We shall speak of NH_3 as undissociated ammonia.

2 ml. to 0.1 ml., care being taken to operate always on the linear portion of the extinction coefficient-concentration calibration curve. This required in many cases dilution of the sample to 104 ml. in place of the usual 52 ml.

The volume was determined on selected groups of a few cells believed to be representative of all the cells used in an experiment.

The pH of the sap was determined by a new method. It has been noticed repeatedly in the past that the pH of the sap rises immediately after extraction. This seems to be connected in part with the loss of carbon dioxide, and the process is hastened by anything which furnishes nuclei on which the gas bubbles can form. Metal surfaces fall in this class and mere passage of the sap through a stainless steel hypodermic needle, during extraction, is sufficient to change the pH appreciably. In the new method the loss of gas was avoided. Tuberculin syringes of pyrex glass, usually of 0.25 ml. capacity, were used. These were fitted with all-glass needles, and each syringe had a small pyrex glass bead in it to mix the contents after the sap and indicator had been drawn in.

The procedure was as follows. With piston pushed in a cell was impaled and its sap was forced into the syringe by squeezing the cell. This carried the small amount of air in the needle into the syringe and also served to wet the barrel and piston and to move the piston out. The piston was then pushed in, expelling the air and filling the needle with sap, which might, however, have lost some of its gas during its brief exposure to the air. Another charge of sap was then forced into the syringe pushing the piston but no air ahead of it. This and one more charge were used to wash out the syringe and the charge of sap to be tested was then forced in. The required amount of indicator was then drawn into the needle and was forced into the barrel by impaling another cell and squeezing the sap into the needle. The charge of sap and indicator, still free of air bubbles was then mixed by means of the glass bead. By this procedure we obtained a sample of sap mixed with indicator, which had not been exposed either to air or to a metal needle after extraction.

The pH could be determined by comparing the test syringe with buffers and indicators in similar syringes, but we wished to keep on using the Hellige double-wedge colorimeter which has been so satisfactory. Accordingly a special carrier was devised to hold the syringe in the colorimeter in approximately the position usually occupied by the conventional glass cell. A simple plano-convex lens was placed between the syringe and the eyepiece. This had the effect of spreading the emergent beam from the syringe so that only a narrow band passing through the middle of the syringe was viewed. Since the syringe is cylindrical the intensity of the color of the band necessarily decreases from the center. But by selecting only a narrow band the decrease in color to the edges of the field of view was made inappreciable.

The ordinary tuberculin syringe has two other disadvantages. The figures etched on the barrel must be kept out of the field by turning the syringe until they are out of way, and the inner surface of the barrel is ground so that even when it is filled with liquid some scattering occurs. This has the effect of slightly reducing the brilliance of the color, but one soon learns to discount this slight effect. Doubtless the difficulty could be overcome by using syringes made of Jena KPG tubing which is so accurately fabricated that barrel and piston do not have to be ground to a fit. The pH's were read off a calibration curve constructed by means of buffers, treated in the syringe in the same way as the unknown sample.

RESULTS

Four experiments comparing the rates of accumulation in normal light[3] and darkness are discussed.

Experiment 1.—Cells were exposed to sea water containing 0.00175 M ammonia at the normal pH of sea water.[4] The results of this experiment are given in Table I and Fig. 1. The behavior of sodium and potassium will be considered in detail. In this experiment the pH was determined parallel with the ammonia changes by the method described in this paper. The results of the pH measurements together with the rate of increase of ammonia have been plotted in Fig. 3. Since the method is rather wasteful of cells the pH was not determined in Experiments 2, 3, and 4.

Experiment 2.—Cells were exposed in light and dark to sea water containing 0.0025 M ammonia at normal pH. The results are given in Fig. 1.

Experiment 3.—Cells were exposed in light and dark to sea water containing 0.0025 M ammonia at normal pH. The dark group was subjected in this experiment to extremely dark conditions. The results are given in Fig. 2.

Experiment 4.—As in Experiment 3, except that before exposure to sea water containing ammonia the cells of both groups were subjected to a preliminary period of darkness in ordinary sea water. (See Fig. 2.)

Before considering the rates of entrance let us consider the pH measurements. From Fig. 3 it appears that soon after the cells were exposed in normal light to the sea water containing ammonia the pH rose by a rather small but quite definite amount (by 0.4 pH), and that the new pH level at pH 6.10 was maintained until the end of the experiment. In darkness also the pH rose promptly though not quite so much as in the light. But in this case a slow decrease then followed and at the end of the experiment the pH of the sap was about that of normal sap, in spite of the fact that the sap now had 0.05 M NH_4^+ and had lost a corresponding concentration of potassium. It appears then that there is little correlation between the concentration of ammonia in the sap and the pH. This is particularly clear in the

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[3] Normal light means the regular daily succession of light and darkness.

[4] The pH of the body of the sea water was 8.2 but it varied in the immediate neighborhood of the cells depending on illumination, etc.

TABLE I

Accumulation of Ammonia in Sea Water Containing 0.00175 M Ammonia (Experiment 1)

Days	Volume	Concentration			P'_L	P'''_L
		Ammonia	Potassium	Sodium		
Light group						
	cc.	M	M	M		
0	1.98	0.0006	0.4846	0.1475	—	
2.5	2.01	0.0274	0.4770	0.1520	0.0039	0.0027
5	2.02	0.0493	0.4420	0.1576	0.0035	0.0026
7	2.04	0.0600	0.4274	0.1600	0.0031	0.0023
10	2.06	0.0850	0.4013	0.1649	0.0031	0.0026
13	2.09	0.0978	0.3780	0.1786	0.0027	0.0024
15	2.10	0.1178	0.3725	0.1692	0.0029	0.0028
20	2.13	0.1339	0.3400	0.1779	—	
26	2.16	0.1319	0.3143	0.1832		
Dark group						
	Adjusted volume*				P'_D	P'''_D
0	1.98	0.0006	0.4846	0.1475	—	—
2.5	1.97	0.0184	0.4766	0.1425	0.0097	0.0076
5	2.00	0.0253	0.4628	0.1524	0.0067	0.0058
7	1.94	0.0286	0.4729	0.1429	0.0077	0.0050
10	1.97	0.0395	0.4760	0.1360	0.0054	0.0062
13	2.01	0.0464	0.4515	0.1516	0.0049	0.0077
15	1.98	0.0477	0.4509	0.1539	0.0043	0.0073
20	2.02	0.0494	0.4361	0.1572	0.0035	0.0067
26	2.02	0.0518	0.4366	0.1415	—	—

* Adjusted by multiplying the measured volume in the dark group by 1.98 ÷ 1.80 so as to make it possible to start light and dark group "moles" curves at the same zero point.

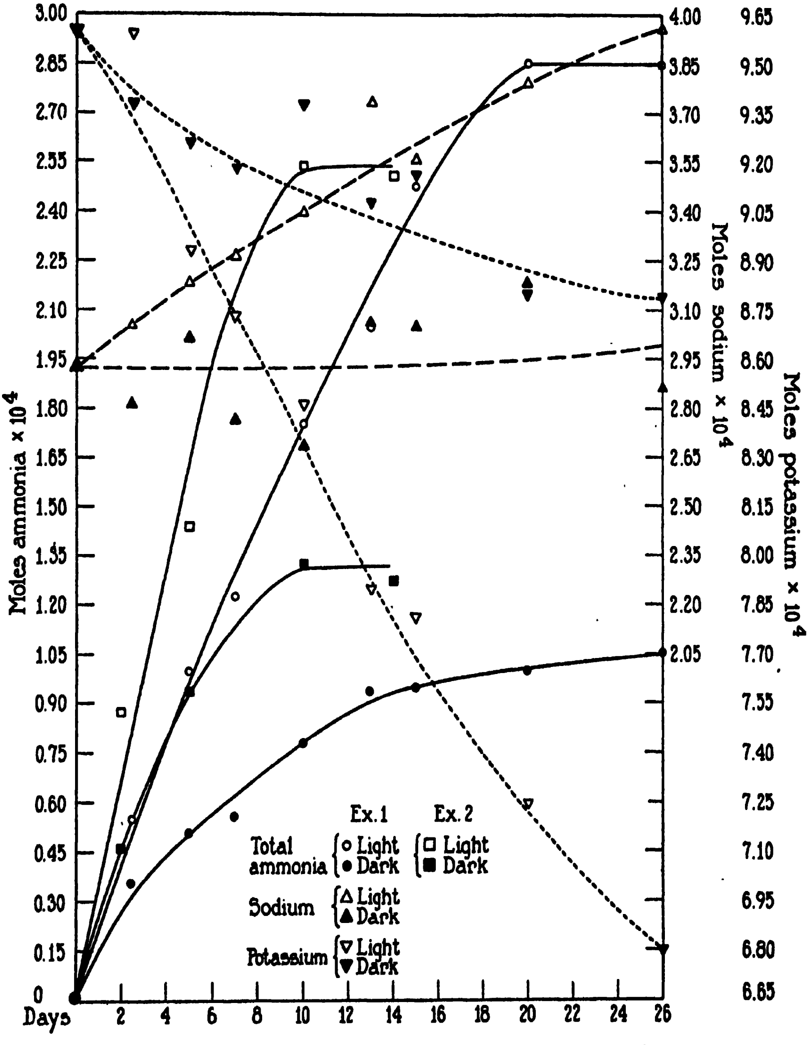

FIG. 1. Accumulation of ammonia in light and darkness. Experiments 1 and 2. The curves are drawn free-hand to give an approximate fit.

dark group where after the first rapid pH rise the ammonia concentration and the pH moved in general in opposite directions.

These results contradict effectively a suggestion made in a former paper that the presence of a relatively high concentration of ammonia in the sap results in the buffering of the sap by ammonium salts at a somewhat higher level than that of normal sap. It now seems much more likely that the change in pH may be associated with relatively small amounts of basic ammonia in the sap.

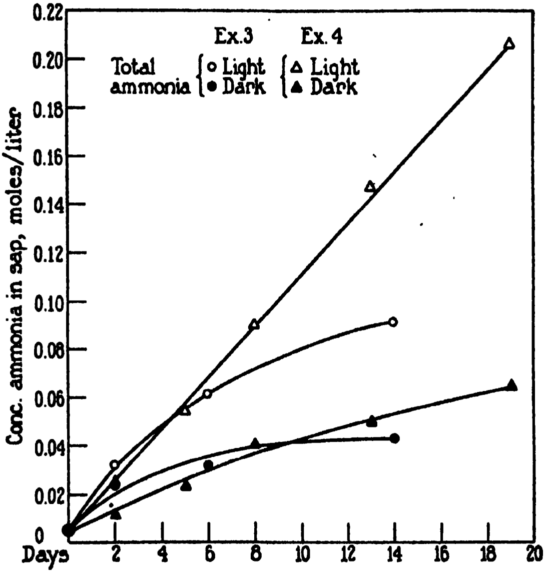

FIG. 2. Accumulation of ammonia in light and extreme darkness. Experiments 3 and 4. The curves are drawn free-hand to give an approximate fit.

In the accumulation of ammonia, tending to raise the pH of the sap, we suppose that we have the entrance of ammonia as NH_4X,[5] a form equivalent to entrance as a base since each molecule of NH_4X is decomposed at the sap-protoplasm interface by an acid stronger than HX (probably CO_2) thus,

$$NH_4^+ + X^- + H^+ + HCO_3^- \rightarrow (NH_4)^+ + HX + HCO_3^-$$

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[5] Osterhout (Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1935, **21**, 125) has suggested that ammonia entrance is preceded by a reaction between ammonia in the sea water and an acid HX in the protoplasm.

Since *HX* is by assumption a very weak acid nearly insoluble in water it does not figure in the pH of the sap. And tending to lower the pH we have (*a*) the loss of potassium as *KX* which is equivalent to the loss of a base, since KOH from the sap unites with *HX* in the protoplasm to form the *KX*. (*b*) The elaboration in the protoplasm of water-soluble acids which pass into the sap. Carbon dioxide is the most conspicuous of these but others probably play a part. This process which goes on constantly is in part offset by the outward diffusion of CO_2 from the sap to the sea water. In the light photosynthesis, which we assume to be confined chiefly to the sea water-protoplasm interface, removes the CO_2 there and as a result the gradient of concentration of CO_2 from the sap to the sea water is increased. This results in a more rapid loss of CO_2.

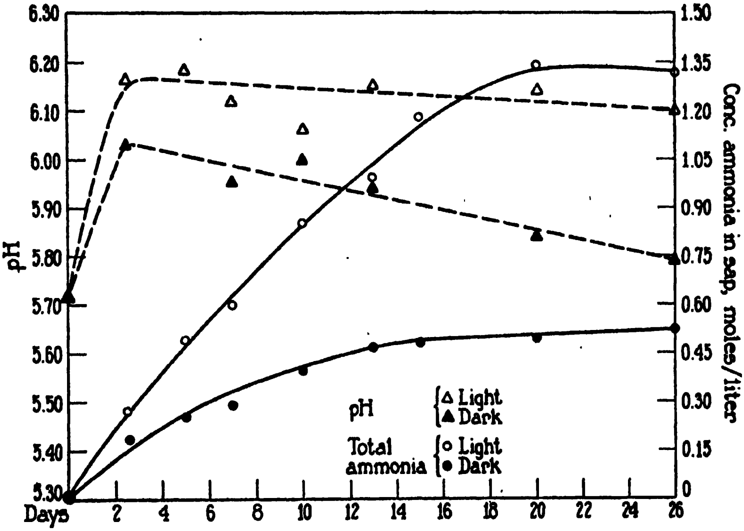

FIG. 3. Change of pH of sap during accumulation in light and in darkness. The curves are drawn free-hand to give an approximate fit.

The pH of the sap is thus the resultant of several processes. The mixture of buffer systems is probably quite complex. But we may say confidently that the increase of free base will raise the pH and the decrease of base will decrease it. Such a change might possibly be brought about by the replacement of KOH by an equivalent amount of NH_3 with the system moving towards a higher pH due to the introduction of a new buffer system. Instead we believe that the results are more readily explained by assuming that the pH rise in both the light and dark groups depends on the rapid entrance of ammonia in a form equivalent to the base, without the loss of a corresponding amount of potassium as base. In the accumulation of ammonia we notice that in general at the very start of the process the total concentration of cations in the sap increases a little. We associate this in part with the gain of some ammonia without an equivalent loss of potassium.

In both groups the same process operates to raise the pH at the start, but in the dark group because the CO_2 diffuses out of the sap more slowly than in the light the effect of the entrance of the excess of ammonia over the potassium lost, tends to be nullified. Hence the slow decrease of pH.

We must now consider to what extent the pH measurements are trustworthy. We have to face the fact that the sap is poorly buffered. For example, probably the buffer system of normal sap is due to sodium bicarbonate and potassium bicarbonate. But the total carbon dioxide concentration of the sap, according to Osterhout and Dorcas,[6] is only 0.0002 M of which, at pH 5.72,[7] one third is salt and the rest free CO_2. The final concentration of indicator used was 0.0001 M, or about half that of the buffer system. It would not be surprising therefore if the buffer equilibrium should be seriously upset by the indicator. A practical test of this possibility was made in New York by determining without gas loss the pH of a sample of sap with two concentrations of indicator. The same value was obtained in both cases. But this might mean that the buffer capacity of the indicator is too great to be upset by the addition of sap. This could hardly have been so in the present case since there was an obvious change in the color of the indicator as it was added to the sap. On the whole therefore we believe that the measurements give a true picture of the trend of the pH changes, and roughly of their magnitude.

[6] Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925–26, 9, 255.

[7] This value was obtained in the present paper. The proportion of salt to base was calculated by the Henderson-Hasselbalch equation, using for pK'_1 the value of 6.02 according to the latest results of MacInnes and Shedlovsky.

Considering now the rate experiments, all four lead to the conclusion that the rate of entrance of ammonia is strongly influenced by light. Thus the rate of entrance of moles (concentration × volume) was about 2.5 times as fast in light as in darkness. This was hardly a question of the greater rate of growth of the cells in light. On a concentration basis in which we ignore the difference in growth rate the rate of concentration increase was about twice as fast in two experiments, and about three times as fast in the other two.

From Experiment 3 we can draw the additional conclusion that accumulation can go on even in total darkness. In this experiment in order to rule out the possibility that any daylight at all might get to the dark group the bottles in which the cells were exposed were covered with thick layers of adhesive tape painted black. These bottles were placed in sea water in a ten gallon stoneware crock with a cover. This was kept in a room sufficiently dark to be used as a photographic dark room. To keep the cells at about the same temperature as the light group, the stoneware crock was immersed in a bath through which a flow of salt water was maintained. In spite of the absence of light the cells accumulated ammonia and the rate was comparable with that observed in Experiment 2 in which the darkness was not so complete. The rate in darkness was about half that in normal light.

The cells of Experiment 3 were transferred directly from a large collection which was kept under normal light. It seemed not impossible therefore that they might have stored energy during this period which would be available for accumulation for a considerable period after the withdrawal of the light. In order to minimize this factor, if it should exist, the cells of Experiment 4 were first subjected to a preliminary exposure to normal sea water in total darkness for 14 days. This treatment had no visible effect on the rate of accumulation in the dark. In the light accumulation took place somewhat faster than in Experiment 3. However, Experiments 3 and 4 are not directly comparable and we cannot say definitely that the preliminary dark treatment rendered the cells capable of accumulating ammonia faster in the light.

All the curves for the accumulation of ammonia in Fig. 1 flatten out towards the end of the exposure. In the light where the cells continue to grow we should expect the moles of ammonia to continue to increase. However, at best the growth is slow and the error of the volume measurements and the natural variation of the sap samples are such that when the rate of ammonia entrance has become slow some uncertainty in the location of the points is to be expected. The simplest interpretation of the flattening is that the concentration of ammonia in the sap has reached a constant level, and that thereafter as the volume increases slowly, ammonia is taken in slowly to keep the concentration constant.

The approach to constant ammonia concentration during ammonia accumulation is also clearly foreshadowed in the curves for Experiment 3 (Fig. 2) and in a curve in a previous paper.[8] But there is little or no flattening of the curves of Experiment 4. This may mean merely that the experiment was terminated before the onset of the flattening. On the other hand it may be connected with the preliminary dark treatment. This point will be the subject of further investigation.

The flattening of the curve suggests the approach to an equilibrium or rather, since we are dealing with a living system, to a "steady state."

For equilibrium we may write that

$$f_o^{\mathrm{NH_3}} [\mathrm{NH_3}]_o = f_i^{\mathrm{NH_3}} [\mathrm{NH_3}]_i$$

where f is the activity coefficient, $[\mathrm{NH_3}]$ is the concentration of undissociated ammonia, and o and i refer to the sea water and sap respectively.

Assuming[9] that $f_o^{\mathrm{NH_3}} = f_i^{\mathrm{NH_3}}$ the equation reduces to

$$[\mathrm{NH_3}]_o = [\mathrm{NH_3}]_i$$

It can be shown that with certain assumptions the same equation should fit the steady state.

The derivation[10] is as follows. According to Osterhout[5] ammonia may pass through the non-aqueous protoplasm as $\mathrm{NH_4}X$ where X is the anion of a weak acid elaborated in the non-aqueous layer of the protoplasm. It is assumed that the reaction whereby NH_4X is formed takes place on the protoplasmic side of the sea water-protoplasm or sap-protoplasm interface. We assume that the protoplasm[11] has the structure shown in Fig. 4. For purposes of discussion we may neglect the aqueous layer *W* and treat the protoplasm as though it consisted of a single non-aqueous layer. Now at each interface there is a pair of adjacent unstirred layers, one in the aqueous phase and the other in the non-aqueous protoplasmic surface layer. And in each pair of such layers there are thin regions immediately in contact where there is equilibrium across the interface. At the sea water interface the aqueous unstirred layer is designated hereafter as *op* and the non-aqueous layer as *po*. The thin equilibrium layers are called *eop* and *epo*. At the sap-protoplasm interface the corresponding designations are *ip*, *pi*, *eip*, and *epi*. (See Fig. 4.)

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[8] Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1930–31, **14**, 301 (Fig. 3).

[9] *Cf.* Zscheile, F. P., Jr., *Protoplasma*, 1930, **11**, 481.

[10] This derivation is given in detail as it will be used as the foundation for calculations in subsequent papers.

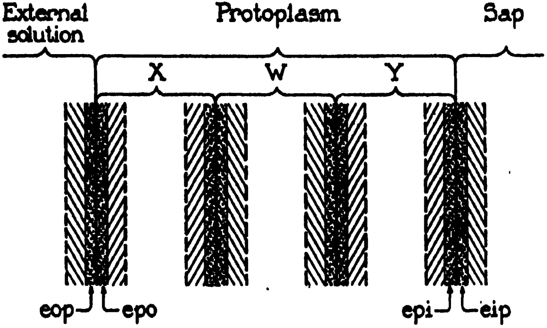

FIG. 4. Hypothetical structure of the protoplasm. *X* and *Y* are non-aqueous layers; *W* is an aqueous layer. The shaded areas represent unstirred layers. The very narrow stippled bands, bounding each interface, are extremely thin layers which are in approximate equilibrium: some of these are labelled; *i.e.*, *eop*, *epo*, *epi*, and *eip*.

Now at the steady state if we ignore the small amount of growth which occurs,

$$[NH_4X]_{epo} = [NH_4X]_{epi}$$

since in this condition the flux of NH_4X, which is assumed to be the only species carrying ammonia, is zero.

But we assume that at each interface some of the NH_4X formed is transferred to the adjacent aqueous layers, so that the following equilibria are set up

$$\left.\begin{aligned} &[NH_4X]_{epo} = S_{eop}^{NH_4X}[NH_4X]_{eop} \\ \text{and} \quad & \\ &[NH_4X]_{epi} = S_{eip}^{NH_4X}[NH_4X]_{eip} \end{aligned}\right\} \qquad (1)$$

where *S* is the partition coefficient.

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[11] Osterhout, W. J. V., *Ergebn. Physiol.*, 1933, **35**, 967.

In the aqueous layers we have the following hydrolytic reaction,

$$NH_4X + H_2O \rightleftharpoons NH_4OH + HX$$

for which we can write the thermodynamic mass action equation,

$$K_{\text{hyd.}} = \frac{(NH_4OH)_{eop}(HX)_{eop}}{(NH_4X)_{eop}(H_2O)_{eop}} = \frac{(NH_4OH)_{eip}(HX)_{eip}}{(NH_4X)_{eip}(HX)_{eip}} \tag{2}$$

where parentheses indicate activities. But

$$NH_4OH \rightleftharpoons NH_3 + H_2O$$

for which the thermodynamic mass action equation is

$$k_h = \frac{(NH_3)_{eop}(H_2O)_{eop}}{(NH_4OH)_{eop}} \tag{3}$$

Now

$$(HX)_{eop} = f_{eop}^{HX}[HX]_{eop} = \frac{f_{eop}^{HX}[HX]_{epo}}{S_{eop}^{HX}}$$

where square brackets indicate concentrations. (A similar set of equations apply to the *eip-epi* interface.)

Whence for the second term of equation (2) we get

$$(NH_4X)_{eop} = f_{eop}^{NH_4X}[NH_4X]_{eop} = \frac{f_{eop}^{NH_4X}[NH_4X]_{epo}}{S_{eop}^{NH_4X}}$$

$$= \frac{[NH_3]_{eop}HX_{epo}\, f_{eop}^{HX}\, f_{eop}^{NH_3}}{K_{\text{hyd.}}\, k_h\, S_{eop}^{HX}} \tag{4}$$

and for the third term of equation (2)

$$\frac{f_{eip}^{NH_4X}[NH_4X]_{epi}}{S_{eip}^{NH_4X}} = \frac{[NH_3]_{eip}[HX]_{epi}\, f_{eip}^{HX}\, f_{eip}^{NH_3}}{K_{\text{hyd.}}\, k_h\, S_{eip}^{HX}} \tag{4a}$$

or collecting constants

$$[NH_4X]_{epo} = K'_{\text{coll.}}[NH_3]_{eop}[HX]_{epo}$$

and

$$[NH_4X]_{epi} = K''_{\text{coll.}}[NH_3]_{eip}[HX]_{epi}$$

Or assuming that corresponding activity and partition coefficients are equal in sap and sea water, at the steady state

$$[NH_3]_{eop}[HX]_{epo} = [NH_3]_{eip}[HX]_{epi} \tag{5}$$

or if there are no concentration gradients of $[NH_3]$ across the *op* and *ip* layers, as is almost certainly the case,

$$[NH_3]_o[HX]_{opo} = [NH_3]_i[HX]_{opi} \tag{5a}$$

Now if HX is distributed equally throughout the non-aqueous protoplasm $[HX]_{opo} = [HX]_{opi}$. For purposes of calculation we may regard $[HX]_{opo}$ and $[HX]_{opi}$ as constant. We then have for the steady state,

$$[NH_3]_o = [NH_3]_i \tag{6}$$

If a steady state has been attained we should be able to upset it by changing $[NH_3]_o$. An experiment to determine if this could be done was carried out by exposing cells which had ceased to accumulate ammonia to (*a*) sea water containing more ammonia at the normal pH of sea water and (*b*) to sea water containing the same amount of total ammonia at a higher pH. There were six groups in this part of this experiment.

(*a*) Light and dark sub-groups in which cells from the light and dark groups of Experiment 1 were exposed respectively in normal light and darkness to sea water at the normal pH[4] containing 0.00175 M ammonium chloride. Since this involved no change in conditions these cells were regarded as controls.

(*b*) Light and dark sub-groups in which cells as in (*a*) were exposed to sea water containing 0.0025 M ammonium chloride at normal pH.

(*c*) Light and dark sub-groups in which cells as in (*a*) were exposed to sea water containing 0.00175 M ammonium chloride at pH 9.5.

As Table II shows, the concentration of ammonia in the sap increased hardly at all in the control groups, but it did increase decidedly in the others. This suggests that real steady states had been attained in both light and dark groups of Experiment 1 during the exposure of the cells to sea water, at normal pH, containing 0.00175 M ammonium chloride, since in both groups increasing $[NH_3]_o$ caused the concentration of total ammonia in the sap to increase. It is noteworthy that in the dark sub-groups (*b*) and (*c*) there is a suggestion of an approach to new steady states. This was not the case in the light sub-groups, but it may have been that in these cases new steady states would have been attained if it had been possible to continue the experiments further.

We may now compare the steady state concentrations attained in Experiment 1 with values calculated on the assumption that at the steady state $[NH_3]_o = [NH_3]_i$.

The normal pH of the sea water is 8.2 and when $[Am]_o$ = total ammonia in sea water = 0.00175 M, $[NH_3]_o = 5.9 \times 10^{-5}$, according to the Henderson-Hasselbalch equation.[13] But the pH of the sap according to the measurements discussed on pages 237 to 242 was about 5.8 at the steady state. At this pH in order for $[NH_3]_i$ to be equal to $[NH_3]_o = 5.9 \times 10^{-5}$, $[Am]_i$ would have to be approximately 0.4 M.

TABLE II

Light group			Dark group		
Sub-group	Days	Concentration ammonia in sap	Sub-group	Days	Concentration ammonia in sap
		M			M
(*a*) Control	0	0.1329	(*a*) Control	0	0.0494
	2	0.1343		2	0.0512
	5	0.1400		5	0.0499
				12	0.0532
(*b*)	0	0.1329	(*b*)	0	0.0494
	2	0.1655			
	5	0.2072		5	0.0704
				12	0.0729
(*c*)	0	0.1329	(*c*)	0	0.0494
	2	0.1500		2	0.0650
	5	0.1815		5	0.0688

Actually it was about 0.05 M. In the light group $[Am]_i$ = 0.13 M which is 6/10 of the theoretical value of $[NH_3]_o = 5.9 \times 10^{-5}$ when pH_o is taken as 8.2. As a matter of fact it is almost certain that during the period of illumination at least, pH_{sw} is greater than pH_o since by photosynthesis CO_2 is removed from the sea water, and although with a large body of sea water in proportion to the cell volume, as was used in our experiments, this effect does not show up in the bulk of sea water, some increase of pH in the unstirred layer of sea water in the cellulose wall adjacent to the protoplasm seems inevitable in light.

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[13] In this calculation pK'_b was taken as 4.34. The reason for this choice is given in a previous paper (Jacques, A. G., *J. Gen. Physiol.*, 1935–36, **19**, 397).

Crozier[13] has shown that in aquaria the photosynthesis of *Valonia* may raise the pH to 9.5 in sunlight and this agrees with our results when the volume of cells to sea water is not too great. Apparently at 9.5 the precipitation of calcium or magnesium carbonates or both serves to buffer the system somewhat by removing $CO_3^=$ ion so that 9.5 is the limit to which pH_{eop} can rise during illumination.

At this pH the maximum value of $[NH_3]_{eop}$ neglecting loss of NH_3 from *eop* to *o* by diffusion would be 7.0×10^{-4}.

But pH_i according to our measurements, in the light group, was 6.10 at the end of the experiment. Hence $[Am]_i$ should be 2.5 M in order for $[NH_3]_i$ to be equal to $[NH_3]_{eop} = 7.0 \times 10^{-4}$. The steady state value of $[NH_3]_i$ corresponding to $[NH_3]_o$ in normal light must be somewhere between 0.21 M and 2.5 M and in any case it is greater than 0.13 M, the value found.

These calculations are only approximate since they depend on the somewhat arbitrarily selected value of 4.34 for pK_b'. However, calculating for the dark group where complications due to photosynthesis are absent the pH which the sap would have to have in order to make $[NH_3]_o = [NH_3]_i$ when $pH_o = 8.2$ and $pH_i = 5.8$, we get $pH_i = 6.7$. The actual value was 5.80 and the discrepancy can scarcely be explained away even by making all possible allowances for error in the pH measurements and error in the selection of the value for pK_b'.

It will be recalled (p. 246) that the conclusion that $[NH_3]_o = [NH_3]_i$ at the steady state is based on the assumptions that (*a*) corresponding activity coefficients in sap and sea water are equal; (*b*) corresponding partition coefficients in sap and sea water are equal, and (*c*) $[HX]_{opo} = [HX]_{opi}$.

The first is almost certainly valid since the ionic strengths of sap and sea water are not far apart. In general we have assumed that (*b*) also is valid, but our information in this respect is vague. But in regard to (*c*) it is not improbable that $[HX]_{opi} > [HX]_{opo}$. Such a situation could arise if *HX* is elaborated only at the sap-protoplasm interface. In this case the equation governing the steady state is equation (5*a*) (p. 247); *i.e.*,

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[13] Crozier, W. J., *J. Gen. Physiol.*, 1918–19, **1**, 581.

$$[NH_3]_o[HX]_{epo} = [NH_3]_i[HX]_{epi}$$

Now according to the calculation for the dark group (p. 248), where complications due to photosynthesis are absent, $[Am]_i$ is only 1/8 of what it should be in order for $[NH_3]_i$ to equal $[NH_3]_o$. But if $[HX]_{epo}$ is only $1/8[HX]_{epi}$, equation (5*a*) will be satisfied; *i.e.*, the ratio $[HX]_{epo} \div [HX]_{epi} = 0.125$.

It is assumed that all the ammonia passing through the protoplasm travels as NH_4X whence the flux for cells with surface of unit area and thickness is given by

$$\frac{d[Am]}{dt} = D^{NH_4X}\{[NH_4X]_{epo} - [NH_4X]_{epi}\} \tag{7}$$

where [Am] means concentration of ammonia: D is a constant for the movement of NH_4X in the protoplasm which is the analogue of a diffusion constant.

When $[HX]_{epo}$ is considered to be equal to $[HX]_{epi}$ and constant this reduces to

$$\frac{d[Am]}{dt} = D^{NH_4X}K'''_{\text{coll.}}[HX]_{epo}\{[NH_3]_{eop} - [NH_3]_{eip}\} \tag{8}$$

$$K'''_{\text{coll.}} = \frac{S^{NH_4X}_{eop}}{S^{HX}_{eop}}\,\frac{f^{HX}_{eop}f^{NH_3}_{eop}}{f^{NH_4X}_{eop}}\,\frac{1}{K_{\text{hyd.}}k_h} \tag{8a}$$

We now proceed to calculate the permeability constants from equation (8). Putting this in a familiar form[14] for simplicity, we have,

$$\frac{dx}{dt} = P'(a - x) \tag{9}$$

where $a = [NH_3]_{eop}$, $x = [NH_3]_{eip}$, and P' contains all the constants of equation (8). On integration

$$P' = \frac{2.3}{t}\log\frac{a}{a - x} \tag{10}$$

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[14] This is, of course, only an approximation in many cases, *cf.* Jacobs, M. H., *Ergebn. Biol.*, 1935, **12**, 1. According to this equation the rate is directly proportional to $NH_{3o} - NH_{3i}$. But this is not necessarily true when the initial rates with different concentrations of NH_{3o} are compared (see footnote 5).

In the present case we have first applied equation (10) to the dark group of Experiment 1 (Table I). a has been calculated on the assumption that the $\mathrm{pH}_o = \mathrm{pH}_{eop} = 8.2$ and $[\mathrm{Am}]_{eop} = [\mathrm{Am}]_o$, whence $[\mathrm{NH_3}]_{eop} = a = 5.9 \times 10^{-5}$ (see p. 248). x has been calculated from $[\mathrm{NH_3}]_{eip}$ on the assumption that $\mathrm{pH}_{eip} = \mathrm{pH}_i = 5.90$. The result is given in Table I under P'_D. It will be seen that this "constant" decreases steadily with time.

If $[\mathrm{H}X]_{epo} \not\gtrless [\mathrm{H}X]_{epi}$, we must put

$$\frac{d[\mathrm{Am}]}{dt} = D^{\mathrm{NH_4}X} K'''_{\mathrm{coll.}} \{[\mathrm{NH_3}]_{eop} [\mathrm{H}X]_{epo} - [\mathrm{NH_3}]_{eip} [\mathrm{H}X]_{epi}\}$$

Putting this in the simplified form

$$\frac{dx}{dt} = P''\{ba - cx\}$$

where $b = [\mathrm{H}X]_{epo}$ and $c = [\mathrm{H}X]_{epi}$. Since we do not know the absolute values of either b or c we multiply the right hand by c/c to get

$$\frac{dx}{dt} = P'''\left(\frac{b}{c}a - x\right)$$

where $P''' = cP''$. On integration we get

$$P''' = \frac{2.3}{t} \log \frac{\frac{b}{c}a}{\frac{b}{c}a - x} \tag{11}$$

This equation has been used to calculate P'''_D. The most natural assumption to make in calculating the coefficient b/c is that at the end of the experiment when the steady state was attained

$$b/c\,[\mathrm{NH_3}]_o = [\mathrm{NH_3}]_i$$

Assuming that $\mathrm{pH}_i = 5.90$ and $[\mathrm{Am}]_i$ at the steady state was 0.0518, $[\mathrm{NH_3}]_i = 9.0 \times 10^{-6}$ and this value was used to calculate the permeability constant for the dark group. By trial we found that the value 8.83×10^{-6} [15] for $b/c\,[\mathrm{NH_3}]_o = [\mathrm{NH_3}]_i$, gave a slightly better series of values for the permeability constant. These values are given as P_D''' in Table I. Although the values are erratic there is no definite trend and the deviations may be associated with faulty sampling of the sap and errors in analysis.

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[15] We may not assume that at the steady state $b/c\,a < x$, but the slight correction from 9.0×10^{-6} to 8.83×10^{-6} is within the limits of the natural variations of the cells.

On using the value 1.0×10^{-5} for b/c $[NH_3]_o$ the values of the permeability constant had a marked trend. Hence we conclude that 8.83×10^{-6} is a significant value.[16] And the ratio b/c is therefore approximately

$$\frac{8.83 \times 10^{-6}}{5.9 \times 10^{-5}} \simeq 0.15$$

In the light group, Experiment 1 (Table I), we may not assume that $pH_{eop} = pH_o$ at least not during illumination. According to Crozier[13] the maximum pH which can be expected due to the photosynthetic removal of CO_2 from the sea water is 9.5. Using this value for pH_{eop} $[NH_3]_{eop} = 1.1 \times 10^{-4}$. But the cells were, in our experiments, illuminated not more than half the time due to the onset of darkness, and in the dark period pH_{eop} may be taken as equal to $pH_o = 8.2$, whence $[NH_3]_o = 5.9 \times 10^{-5}$. The average of these two values $= 3.85 \times 10^{-4}$ has been taken as the value for a in calculating P_L' from equation (10) and x has been calculated on the basis that $pH_i = 6.15$. As Table I shows, P_L' calculated in this way has an obvious drift.

We now apply equation (11) using $b/c = 0.15$ as in the dark group. $b/c\ a$ then equals 5.78×10^{-5} M.

The permeability constant has been calculated on this basis. It was found to be nearly without trend but with a slight upward drift at the end. A slightly better constant was obtained by using for b/c a, 5.9×10^{-5}. This is P_L''' in Table I.

Since by using the factor $b/c = 0.15$ we obtain fairly satisfactory permeability constants we assume that this ratio may have some physical significance. Provisionally we suppose that it is the ratio of $[HX]_{epi}/[HX]_{epo}$. The actual concentration of each is unknown.

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[16] It might be suggested that since the pH value of the sap used in these calculations, *viz.* 5.90, is a compromise value, being the apparent average value of the pH during the entrance of ammonia, that we could correct $[NH_3]_i$ from 9.0×10^{-6} to 8.83×10^{-6} by assuming a slightly lower value for pH_i. But if this corrected value is used in calculating x the constants are the same as those obtained by using 9.0×10^{-6} and $pH_i = 5.99$.

If b/c has the significance suggested at the steady state x should be equal to $b/c\ a$. In the dark group x_e, where e signifies the steady state, was found to be equal to 9×10^{-6}, but $b/c\ a$ was taken as 8.83×10^{-6}. This slight discrepancy is of no importance. In the light group conditions are more complicated, because owing to photosynthesis a is not well known. But by taking a as the probable average value of $[NH_3]_{eop}$ in light and in darkness a value for $b/c\ a = 5.9 \times 10^{-5}$ M was obtained. This value gave a good series of values for the constant but x_e in the light was only 4.1×10^{-5}. Although the two values are of the same order there is some discrepancy here not yet explained.[17]

It appears that P_D''' is about 3 times P_L'''. Assuming that $P_D'' = P_L''$ we may suppose that $[HX]_{epi} = c$ is 3 times as great in the dark. This seems possible for we must remember that some of the HX is being lost to the sea water and this loss may well be much greater in light than in darkness due to the higher pH at the sea water-protoplasm interface when the cell is photosynthesizing actively. We assume that $[HX]_{epo}$ is also 3 times as great in the dark.

Hitherto we have assumed that the reaction is a simple reversible neutralization

$$NH_4OH + HX \rightleftharpoons NH_4X + H_2O$$

But it might be much more complex than this. This will be discussed in a forthcoming paper.

SUMMARY

The accumulation of ammonia takes place more rapidly in light than in darkness. The accumulation appears to go on until a steady state is attained. The steady state concentration of ammonia in the sap is about twice as great in light as in darkness. Both effects are possibly due to the fact that the external pH (and hence the concentration of undissociated ammonia) outside is raised by photosynthesis.

Certain "permeability constants" have been calculated. These indicate that the rate is proportional to the concentration gradient across the protoplasm of NH_4X which is formed by the interaction of NH_3 or NH_4OH and HX, an acid elaborated in the protoplasm. The results are interpreted to mean that HX is produced only at the sap-protoplasm interface and that on the average its concentration there is about 7 times as great as at the sea water-protoplasm interface. This ratio of HX at the two surfaces also explains why the concentration of undissociated ammonia in the steady state is about 7 times as great in the sea water as in the sap. The permeability constant P''' appears to be greater in the dark. This is possibly associated with an increase in the concentration of HX at both interfaces, the ratio at the two surfaces, however, remaining about the same.

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[17] A slight uncertainty in the determination of pH_i at the steady state would account for the discrepancy, but since x and x_e are calculated from the same value of pH_i, the calculated value of the constant would again show large deviations if pH_i were increased enough to make $x_e = 5.9 \times 10^{-5}$.

The pH of sap has been determined by a new method which avoids the loss of gas (CO_2), an important source of error. The results indicate that the pH rises during accumulation but the extent of this rise is smaller than has hitherto been supposed.

As in previous experiments, the entering ammonia displaced a practically equivalent amount of potassium from the sap and the sodium concentration remained fairly constant.

It seems probable that the pH increase is due to the entrance of small amounts of NH_3 or NH_4OH in excess of the potassium lost as a base.

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THE KINETICS OF PENETRATION

XVII. THE EXIT OF AMMONIA IN LIGHT AND DARKNESS

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In a previous paper[1] dealing with the exit of ammonia[2] from cells of *Valonia macrophysa*, Kütz., it was suggested that the rate may be greater in light than in darkness and that the exit is preceded by an induction period. In the present paper these points have been studied in more detail. And at the same time the pH changes of the sap during the exit have been studied by a new method.[3]

In this paper, "exposure to light" means exposure to the normal alternation of daylight and darkness in the laboratory; "dark" means continuous darkness.

EXPERIMENTAL

These experiments were carried out in Bermuda in the winter of 1937-38 at the Bermuda Biological Station.

Cells which had been allowed to accumulate ammonia in the light were divided into two groups and were exposed to a flow of normal sea water, which is nearly ammonia-free, one group in darkness and the other in light.

The cells were exposed in glass jars in the light groups and in darkened glass jars or porcelain jars in the dark groups as described in the previous paper. The jars were closed with two layers of heavy opaque rubber sheeting through which were thrust two glass tubes for the entrance and exit of the flow of sea water. There may have been some slight leakage of light into the dark jars from this cause, but this must have been very small since the tubes themselves were blackened.

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[1] Jacques, A. G., *J. Gen. Physiol.*, 1937-38, **21,** 775.

[2] Ammonia means, as in previous papers, $NH_3 + NH_4OH + NH_4$ ion; *i.e.*, the total amount found by analysis.

[3] Jacques, A. G., *J. Gen. Physiol.*, 1938-39, **22,** 501.

Volumes were determined on small groups of cells assumed to be characteristic of the whole groups by a method previously described.[4] Analyses for potassium, sodium, and ammonia were carried out as described in a recent paper. For the pH of the sap we used the new technique recently described[1] in which the loss of gas, chiefly CO_2, from the extracted sap is avoided.

TABLE I

Exit of Ammonia from Valonia in Light and Dark (Experiment 1)

Days	Volume	Concentration			Moles × 10^4		
		Ammonia	Potassium	Sodium	Ammonia	Potassium	Sodium
				Light group			
	cc.	M	M	M			
0	1.72	0.1260	0.3617	0.1426	2.167	6.221	2.452
2	1.73	0.1270	0.3547	0.1397	2.197	6.136	2.416
5	1.76	0.1188	0.3576	0.1432	2.090	6.294	2.520
10	1.76	0.1186	0.3699	0.1384	1.987	6.520	2.436
15	1.78	0.0863	0.3668	0.1587	1.536	6.529	2.824
21	1.82	0.0739	0.3568	0.1783	1.345	6.493	3.235
26	1.86	0.0615	0.3336	0.2195	1.144	6.204	4.072
				Dark group			
	Adjusted volume*						
0	1.72	0.1260	0.3617	0.1426	2.167	6.221	2.452
2	1.73	0.1279	0.3583	0.1342	2.212	6.198	2.311
5	1.67	0.1345	0.3587	0.1292	2.246	5.990	2.158
10	1.70	0.1154	0.3662	0.1514	1.962	6.225	2.563
15	1.70	0.1168	0.3534	0.1539	1.984	6.007	2.616
21	1.73	0.1025	0.3335	0.1738	1.906	5.769	3.007
26	1.78	0.0905	0.3433	0.1933	1.738	6.110	3.441

* Adjusted by multiplying the measured volume in the dark group by 1.72 ÷ 1.85 so as to make it possible to start light and dark group "moles" curves at the same zero point.

Three experiments were performed.

Experiment 1.—The cells were exposed to sea water containing 0.0025 M ammonium chloride for 14 days in light. Then they were divided into two groups, one in darkness and the other in light, and kept in flowing normal (nearly ammonia-free) sea water for 26 days

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[4] Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1931–32, 15, 537.

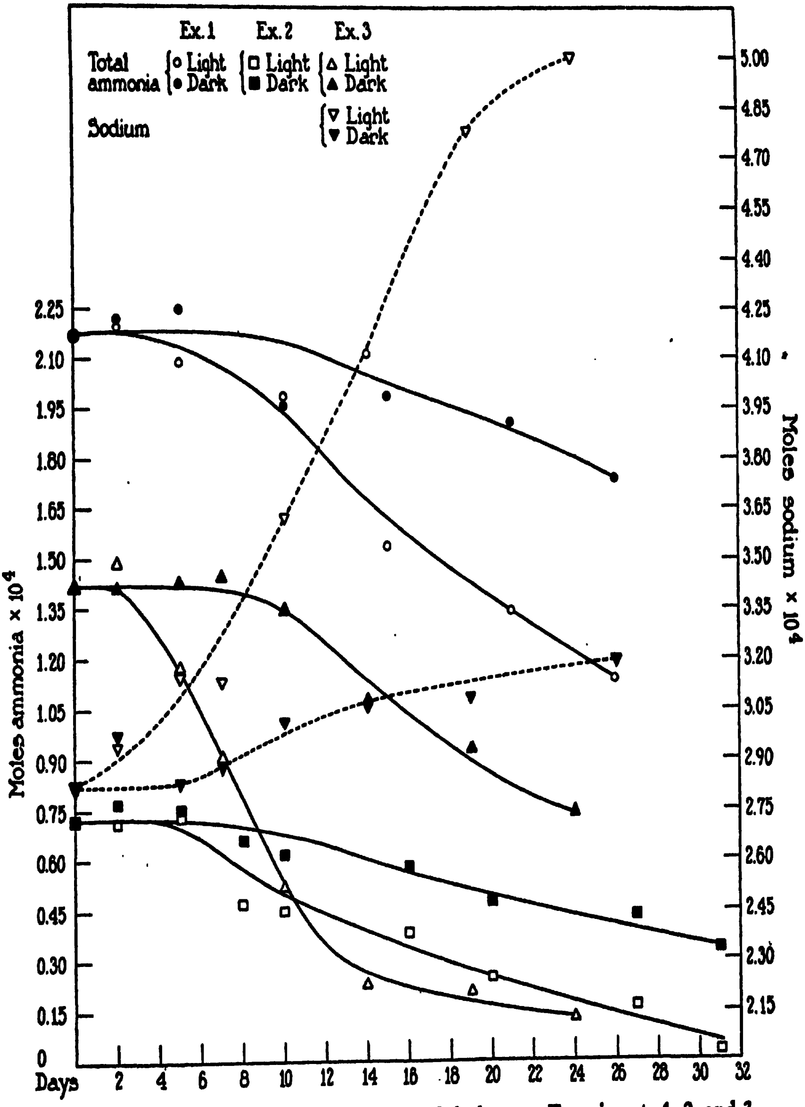

FIG. 1. The exit of ammonia in light and darkness. Experiments 1, 2, and 3, and the entrance of sodium in light and darkness in Experiment 3. The curves are drawn free-hand to give an approximate fit.

in light. The results of this experiment are given in Table I and in Fig. 1.

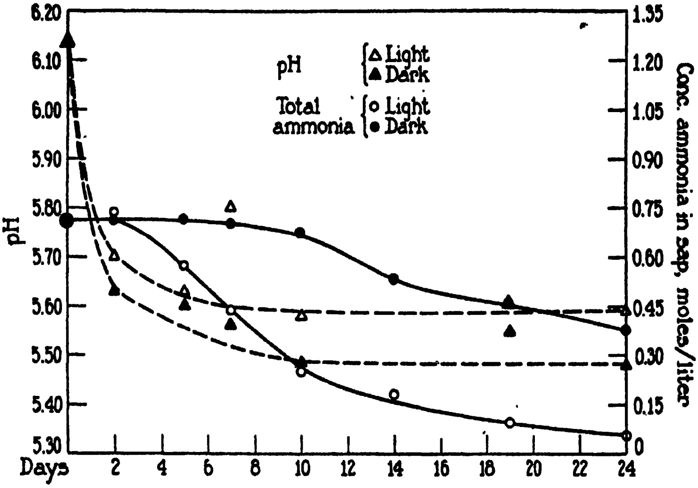

FIG. 2. Comparison of the decrease of ammonia concentration and the change of pH of the sap in light and darkness. The curves are drawn free-hand to give an approximate fit.

TABLE II

Initial and Final Concentrations

Experiment		Stage 1 Before accumulation	Stage 2 At end of accumulation	At end of exit	
				Light group	Dark group
		M	M	M	M
1	Ammonia	0.0005	0.1260	0.0615	0.0905
	Potassium	0.4953	0.3617	0.3336	0.3433
	Sodium	0.1348	0.1335	0.2195	0.1933
2	Ammonia	0.0005	0.0615	0.0017	0.0245
	Potassium	0.4954	0.4464	0.4427	0.4106
	Sodium	0.1297	0.1304	0.1836	0.1906
3	Ammonia	0.0007	0.0707	0.0057	0.0372
	Potassium	0.4957	0.4274	0.3953	0.4130
	Sodium	0.1430	0.1390	0.2210	0.1596

Experiment 2.—The cells were first allowed to accumulate ammonia from sea water containing 0.00100 M ammonium chloride for 13 days

in light. After this some were exposed in light and others in darkness to normal sea water for 31 days. The results are given in Fig. 1.

Experiment 3.—The cells were allowed to accumulate ammonia from sea water containing 0.00175 M ammonium chloride for 21 days in light. Afterwards some were exposed in light and others in darkness to normal sea water for 24 days. The results are given in Figs. 1 and 2 which show the decrease in concentration of ammonia for both light and dark groups during the exposure to normal sea water in comparison with the pH change during exit. For comparison see also Fig. 3 of a previous paper[3] (plotted on the same scale) showing how the pH behaves during accumulation.

Table II gives a summary of the behavior of ammonia, potassium, and sodium, in Experiments 1, 2, and 3, during the accumulation and exit of ammonia.

DISCUSSION OF RESULTS

It appears that during exposure to sea water nearly free from ammonia the exit of ammonia is in all cases preceded by an "induction period" during which the concentration of ammonia either remains constant or the decrease is so slow that it is masked by the natural variations among the cells. The induction period is longer in the dark, and when at length the exit of ammonia occurs at a measurable rate, the loss proceeds more rapidly in the light than in the dark. Eventually, however, when the residual concentration of ammonia in the sap has reached a low value the rate of exit decreases again. Thus the curve has an S shape. The S shape, however, is not clearly apparent in the curves for the exit in the dark, but this may well be because the rate of exit was slow with the result that either the S shape was masked by the natural variations among the cells or it had not appeared by the time the experiments were concluded.

As in the previous experiments[5] the ammonia lost was replaced largely by sodium. A precise idea is hard to get because of the natural variation in the K/Na ratio of different groups of cells. However, we may say with a fair degree of certainty that in 5 of the 6 cases studied when ammonia left the sap the sodium concentration increased and the potassium concentration remained quite constant. It is not always possible to equate the moles of sodium gained to the moles of ammonium lost. This is partly owing to the natural variations, and partly perhaps to a tendency for the total cation concentration of the sap to decrease during exit.[5] This may mean that ammonia is lost at a slightly greater rate than sodium is gained. In one experiment, the light group of Experiment 6, the cell gained moles of potassium also during the exit of ammonia.

However, in this experiment a new situation was encountered, since in the last 11 days of the 31 day exposure to normal sea water, the rate of growth was much greater than during the first 20 days. Thus in the first 20 days the increase in volume was only about 6 per cent, but in the 11 day period it was about 20 per cent. This seems to be chiefly because during the last part of the experiment the cells commenced to bud actively. The sap samples came from both buds and parent cells, since only by including both could we get fair values for the moles of ammonia in the sap. Both buds and parent cells were necessarily included in the volume measurements. But the bud cells not having been exposed to the sea water containing ammonia may not have had any ammonia in them unless they took it from the mother cell. Perhaps such bud cells are able to take up potassium preferentially as do cells which have not been exposed to ammonia. Their inclusion in the sample of sap would therefore tend to raise the potassium concentration of the sample, hence it is not settled by this experiment whether cells which have once accumulated ammonia can again take up potassium. Even in the previous experiment[1] where we seemed to have part of the ammonia replaced by potassium, it is not impossible that the result may have been influenced by the inclusion of buds.

In any case we may say that when ammonia leaves the cell it tends to be replaced by sodium alone.

In the present series of experiments the cells were tested for their ability to reaccumulate ammonia after some of the ammonia had been replaced by sodium. In some cases two accumulations and two partial removals of ammonia were carried out. In all cases the cells were able to reaccumulate ammonia. Some of these experiments are described below.

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[5] Conversely when ammonia is being accumulated actively the total cation concentration generally rises above normal.

In one of them cells from the light group of Experiment 1, Table I, were divided into two sub-groups, and exposed to sea water containing 0.0025 M ammonia, for 8 days (*a*) in light, and (*b*) in darkness. The analyses follow:

	Initial (*a*) and (*b*)	(*a*) after 8 days (light)	(*b*) after 8 days (dark)
	M	M	M
Ammonia	0.0615	0.1565	0.0628
Potassium	0.3336	0.2448	0.3271
Sodium	0.2195	0.2295	0.2185
Total	0.6146	0.6308	0.6084

In this experiment the cells took up ammonia rapidly in the light, and while the concentration of ammonia was rising by 0.0950 M, the concentration of potassium decreased by 0.0888 M. In the dark subgroup, however, all concentrations remained practically unchanged.

The cells of the (*b*) sub-group were then exposed to the same ammoniated sea water in light, and the cells of (*a*) sub-group to a flow of normal nearly ammonia-free sea water in light, both for 28 days. The following results were obtained:

	(*a*) 28 days in normal sea water. In light	(*b*) 28 days in sea water containing 0.0025 M ammonia. In light
	M	M
Ammonia	0.0257	0.1957
Potassium	0.3308	0.2509
Sodium	0.2601	0.1890
Total	0.6166	0.6356

In the (*a*) part of the experiment (in normal sea water) the ammonia concentration decreased by 0.1308 M, but the sodium concentration increased only by 0.0306 M, while the potassium concentration increased by 0.0860 M. This is fairly good evidence that in some cases at least the ammonia lost can be replaced by potassium. The increase in the potassium concentration seems too great to be due to faulty sampling, although in this case errors due to this source were probably aggravated because only a few cells were available at this stage for each sample.

In the (*b*) sub-group the ammonia concentration increased by 0.1329 M, but the potassium decreased by only 0.0762 M. This may be due in part to faulty sampling, but undoubtedly another factor is the marked increase in the total cation concentration which occurred during the reaccumulation. The excess concentration may well be ammonia accumulated without the loss of a corresponding amount of potassium.

Since the (*b*) group cells were able to accumulate ammonia in the light we may suppose that they failed to do so in the dark because the internal ammonia concentration was already at the steady state equilibrium corresponding to the external concentration of ammonia in the dark. In this connection it may be said that in Experiment 1 of a previous paper,[3] where the cells were also exposed to sea water containing 0.0025 M ammonia in the dark, the ammonia concentration in the sap ceased to rise when it reached 0.069 M, which is within reasonable distance of the value 0.063 M found in the present experiment.

Finally the remaining cells of sub-group (*a*) were again exposed to 0.0025 M ammonia sea water, for 5 days in light. The result was as follows:

	Initial	After 5 days in sea water containing 0.0025 M ammonia sea water
	M	M
Ammonia	0.0257	0.1014
Potassium	0.3308	0.2946
Sodium	0.2601	0.2476
Total	0.6166	0.6436

In this case the concentration of ammonia rose 0.0757 M but the potassium concentration decreased by only 0.0362 M. Most of the difference is accounted for by the rise in the total cation concentration by 0.0270 M.

In another experiment the cells were first exposed to sea water containing 0.001 M ammonium chloride for 13 days. They were then transferred to running normal sea water for 33 days. Then they were exposed to sea water containing 0.00175 M ammonium chloride for 15 days, and finally again to running normal sea water for 12 days. All these exposures were in light. The results were as follows:

	Initial	Exposed to 0.001 M ammonia S.W. 13 days	Exposed to normal S.W. 33 days	Exposed to 0.00175 M ammonia S.W. 15 days	Exposed to normal S.W. 12 days
	M	M	M	M	M
Ammonia	0.0005	0.0508	0.0045	0.1700	0.0948
Potassium	0.4949	0.4462	—	0.2843	0.2616
Sodium	0.1297	0.1484	—	0.1626	0.2506
Total	0.6251	0.6454	—	0.6169	0.6070

Although the experiment lasted 73 days, those cells which were allowed to survive to the end of it, after two exposures to sea waters containing ammonia, and two partial removals of ammonia in normal sea water, were apparently uninjured. In the course of the experiment K/Na was reduced from 3.8 to 1.

We may now consider the results of the pH measurements. As Fig. 2 shows, there is apparently very little correlation between the decrease in the pH and the loss of ammonia. Thus in both the light and dark groups the pH dropped promptly from above 6 to between 5.60–5.70. In the case of the light group this was nearly the entire drop during the experiment. In the dark group there was a further drop of perhaps 0.15 pH unit on the average, but this appeared to occur before the end of the induction period. These results reinforce a conclusion drawn in a previous paper[3] that even when there is considerable ammonia in the sap the buffer system of the sap is not an ammonium-salt-ammonia one which tends to maintain the pH above that of the normal sodium (potassium) bicarbonate-CO_2 system. Instead, the change in pH during ammonia accumulation seems to be connected with the gain of a little free ammonia or ammonium hydroxide in excess of the potassium lost, and in exit with the loss of a little more ammonia or ammonium hydroxide in excess of the sodium gained.

It might be suggested that since exit is preceded by an induction period there is no evidence that any basic ammonia is lost as soon as the cell is exposed to ammonia-free sea water. However, only a little ammonia would have to be lost to produce the fall in pH observed, since the buffer capacity of the sap is very low. Such an amount might be lost without appearing in the analyses since it could be smaller than the natural variations among the cells. The induction period instead of being a period of no loss might be a period during which the rate of loss is so small that it is masked by natural variations.

The greater decrease in pH in the dark group might be due to the fact that in the dark none of the CO_2 produced by the protoplasm is used up photosynthetically.[6]

According to a previous paper[5] the rate of accumulation is given by the formula[7]

$$\frac{d[\text{Am}]}{dt} = D^{NH_4X} K'''_{\text{coll.}} \{[HX]_{epo}[NH_3]_{eop} - [HX]_{epi}[NH_3]_{eip}\}$$

where [Am] is the ammonia in the sap, square brackets represent concentrations, and HX is a weak acid elaborated by the protoplasm: *eop* and *epo* refer respectively to the adjacent equilibrium layers in sea water and protoplasm where all species are in equilibrium across the interface, and *eip* and *epi* refer to a corresponding pair of adjacent layers at the sap-protoplasm interface. In the light the pH in the *eop* layer, owing to the photosynthetic removal of CO_2, is greater than in darkness, consequently $[NH_3]_{eop}$, the concentration of undissociated ammonia in *eop*, is greater and so the rate of accumulation is greater.

But when we place the cells in normal sea water which is almost ammonia-free, the direction of the gradient represented by the term inside the brackets is reversed. Consequently ammonia should leave the cell and there is no apparent reason for an induction period. Of course, the pH of the sap drops as soon as the cell is exposed to the ammonia-free sea water and this would reduce $[NH_3]_{eip}$. But even so the direction of the gradient is still outward.

Before going on to a discussion of possible reasons for the induction period we may inquire if injury plays any part.

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[6] We suppose that photosynthesis occurs chiefly at the sea water-protoplasm interface but this must increase the gradient of CO_2 from the sap to the sea water and CO_2 will be lost faster by the sap.

[7] The basic equation is

$$\frac{d[\text{Am}]}{dt} = D^{NH_4X} \{[NH_4X]_{epo} - [NH_4X]_{epi}\}$$

which is the equation for the diffusion of the species NH_4X a molecule produced by the reversible reaction

$$NH_4OH + HX \rightleftharpoons NH_4X + H_2O$$

We might suppose that the cell has a definite trapping mechanism, capable of retaining ammonia and possibly other cations in the sap indefinitely as long as the mechanism is unimpared. From Fig. 1 it appears that in some cases not only did the concentration fail to increase during the last days of accumulation, but there is a suggestion that it decreased slightly. Although we consider the apparent decreases as due to natural variations among the cells, it might be that ammonia has started to come out as the result of injury. On this basis we should not expect an induction period. On the other hand, we might suppose that the cell is normal at the start of the washing out process and retains the ammonia until injury occurs. But if the cell is not injured during exposure to sea water containing ammonia we should not expect injury to occur when it is placed in normal sea water.

The displacement of ammonia by sodium instead of by potassium which is observed, would be expected if the protoplasm were injured so as to be permeable to all the molecular species of the sap and sea water. But as this would take place by simple diffusion there is no reason why it should take place more rapidly in the light as it undoubtedly does. Nor should we expect injury to set in sooner in the light. Moreover we should expect potassium to be lost also, and probably more rapidly than ammonia, for the concentration of potassium chloride in the sap was in all cases much greater than that of the ammonium chloride, but their diffusion coefficients are roughly equal.[8]

These arguments lead to one conclusion, namely that injury is not an important factor in the exit of ammonia.

In looking for reasons for the induction period we come to the possibility that ammonia is transferred between the sap and sea water by some carrier other than the NH_4X discussed in the previous paper.[6,7] The obvious possibilities are undissociated ammonia, the derivatives of carbon dioxide and ammonia, such as ammonium carbonate, ammonium bicarbonate, and ammonium carbamate, and ammonium chloride. At the start of the exit experiments there should be an outwardly directed gradient for all of these species. But there is no apparent reason why there should be an induction period in the exit of any of them. Indeed at the very beginning, before the pH drop in the sap has occurred, we should expect the greatest rate of exit and not an induction period. The decrease in the pH observed at the start of the exposure to normal sea water would be expected in all but the case of NH_4Cl to decrease the rate of movement of the species, and in the latter case there should be no effect of pH at all.

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[8] International Critical Tables, McGraw-Hill Book Company, Inc., New York, 1929, 5, 65, 68.

The rate of exit in the case of undissociated ammonia would be given by the equation,

$$\frac{d\,NH_3}{dt} = K_1\,([NH_3]_i - [NH_3]_o) \qquad (a)$$

for ammonium bicarbonate,

$$\frac{d\,NH_3}{dt} = K_2\,([NH_4^+]_i\,[HCO_3^-]_i - [NH_4^+]_o\,[HCO_3^-]_o) \qquad (b)$$

for ammonium carbamate,

$$\frac{d\,NH_3}{dt} = K_3\,([NH_4^+]_i\,[NH_2COO^-]_i - [NH_4^+]_o\,[NH_2COO^-]_o) \qquad (c)$$

for ammonium carbonate,

$$\frac{d\,NH_3}{dt} = K_4\,([NH_4^+]_i\,[NH_4^+]_i\,CO_3^{=}]_i - [NH_4^+]_o\,[NH_4^+]_o\,[CO_3^{=}]_o) \qquad (d)$$

and for ammonium chloride,

$$\frac{d\,NH_3}{dt} = K_5\,([NH_4^+]_i\,[Cl^-]_i - [NH_4^+]_o\,[Cl^-]_o) \qquad (e)$$

A drop in the pH of the sap, should decrease (*a*) by decreasing $[NH_3]_i$, and also (*b*), (*c*), and (*d*) by decreasing the concentration of the anion. There should be little or no change in (*e*) because the concentration of ammonium is comparatively great.

We may now consider whether ammonia may not be transported from the sap by some other species than a simple salt. The slow formation[9] of this substance might account for the induction period. A possible species might be urea, which does as a matter of fact occur rather widely in plants.[10]

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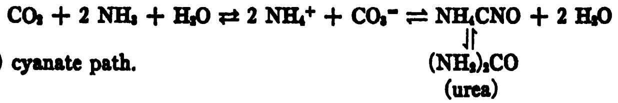
[9] The formation of carbamate from carbonate, may be a comparatively slow reaction, at great dilution (Faurholt, C., *J. chim. phys.*, 1925, **22**, 1) but in the pH range of the sap, according to Faurholt's figures, the proportion of carbamate which can exist in equilibrium with other CO_2-NH_3 species is almost infinitely small.

[10] For a list of occurrences see Handbuch der Pflanzenanalyse (G. Klein), III IV/2, p. 224, J. Springer, Vienna, 1933.

The establishment of the equilibrium in solution between urea and other CO_2-NH_3 species is slow even at high temperatures, but with catalysts it can set up rapidly even at ordinary temperatures. Urease which also occurs in plants is a catalyst for the reaction, which seems to be reversible in its effects. That is to say in a urea solution, urease will bring about decomposition until the equilibrium point is reached, or in a solution of "ammonium carbonate"[11] it will bring about the synthesis of urea.[12]

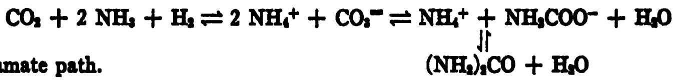
The nature of the reaction is in doubt. Werner[13] believes that the formation of cyanate is a necessary intermediate step in both synthesis and decomposition. But others assume that the reaction proceeds through the rearrangement of ammonium carbonate to ammonium carbamate with the loss of water, and then by the dehydration of the ammonium carbamate to urea.

The two views may be written schematically,



$$\begin{array}{rl} CO_2 + 2\,NH_3 + H_2O \rightleftarrows 2\,NH_4^+ + CO_3^- \rightleftharpoons & NH_4CNO + 2\,H_2O \\ & \downarrow\uparrow \\ & (NH_2)_2CO \\ & \text{(urea)} \end{array}$$

(Werner) cyanate path.



$$\begin{array}{rl} CO_2 + 2\,NH_3 + H_2 \rightleftharpoons 2\,NH_4^+ + CO_3^{=} \rightleftharpoons & NH_4^+ + NH_2COO^- + H_2O \\ & \downarrow\uparrow \\ & (NH_2)_2CO + H_2O \end{array}$$

Carbamate path.

The evidence is contradictory.

Sumner, Hand, and Holloway[14] found that no cyanate was formed when urea was decomposed in the presence of a very pure crystallized urease. Mack and Villars[15] found cyanate among the decomposition products but concluded that only the reaction by way of carbamate is catalyzed by urease. Fearon[16] has recently confirmed Sumner's results, but he suggests that the cyanate path may be an alternative one, and that possibly it is catalyzed by another enzyme present in crude preparations of urease.

For present purposes it is only necessary to assume that there is present in the sap an enzyme capable of facilitating the urea equilibrium. The path is not important. It is a well established fact that many enzymes, including urease, operate most effectively at an optimum pH.

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[11] Ammonium carbonate solution contains, according to the pH, bicarbonate and carbamate ions also.

[12] For recent syntheses of urea see Mack, E., and Villars, D. G., *J. Am. Chem. Soc.*, 1923, **45**, 501. Kay, H. D., *Biochem. J.*, London, 1923, **17**, 277. Fearon, W. R., *Biochem. J.*, London, 1936, **30**, 1652.

[13] Werner, E. A., The chemistry of urea, London, Longmans, Green and Co., 1923.

[14] Sumner, J. B., Hand, D. B., and Holloway, R. G., *J. Biol. Chem.*, 1931, **91**, 333.

[15] Mack, E., and Villars, D. G., *J. Am. Chem. Soc.*, 1923, **45**, 501.

[16] Fearon, W. R., *Biochem. J.*, London, 1936, **30**, 1652.

In the urea-urease system, which unfortunately has been studied mostly with urea as the starting point, the equilibrium is set up most rapidly at pH 7.0 according to the results of Van Slyke and Zacharias.[17] However, the addition of neutral salts or dilution of the urea solution causes the optimum pH to increase. This shift has been confirmed by Lövgren.[18] Recently, however, Howell and Sumner[19] have shown that though an optimum pH exists it depends not only on the concentration of urea but even more so on the type of buffer used in the system. In phosphate buffers the optimum may be as high as 7.6, but in acetate the optimum in the same urea concentration is as low as 6.7. The authors show in the case of acetate buffers that the enzyme is still active at pH's as low as 3.0 and as high as 7.5. In phosphate buffers the range is from 5.0 to 9.

If urea is concerned in the loss of ammonia from the sap it may perhaps operate in the following way. The entering ammonia forms NH_4X at the outer surface and this diffuses into the sap where it is transformed to ammonium chloride. We assume that NH_4Cl cannot diffuse out rapidly since we know from experiments that it cannot diffuse in rapidly.[20] We suppose therefore that ammonia goes out chiefly as urea, the formation of which goes on in such fashion as to explain the induction period.

The urea is formed from CO_2–NH_3 species in the sap in the presence of an enzyme. At the moment the cells are transferred from ammonia sea water to normal sea water the pH of the sap is higher than normal and nearer the optimum pH of the enzyme, consequently urea is formed rapidly and diffuses out of the sap.[21] This is the equivalent of removing ammonium carbonate from the sap since

$$2\,NH_3 + CO_2 \rightarrow (NH_2)_2CO + H_2O$$

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[17] Van Slyke, D. D., and Zacharias, G., *J. Biol. Chem.*, 1914, **19**, 181.

[18] Lövgren, S., *Biochem. Z.*, Berlin, 1921, **119**, 215.

[19] Howell, S. F., and Sumner, J. B., *J. Biol. Chem.*, 1934, **104**, 619.

[20] Cooper, W. C., Jr., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1930–31, **14**, 117.

[21] During the accumulation of ammonia we suppose that the loss of urea goes on, but in this case more ammonia is entering as NH_4X. This is decomposed by carbonic acid and thus keeps the pH up.

hence the pH is decreased. As pointed out previously only a very small amount of ammonia need be lost in this first process to lower the pH. The effect of lowering the pH is to make the enzyme less active so that a comparatively long time elapses before sufficient urea is formed to cause the ammonia concentration of the sap to decrease at a measurable rate. Hence an induction period is observed.

If we assume that the entrance of ammonia depends on the inward diffusion of NH_4X, and the exit of ammonia on the outward diffusion of urea, we run into a number of difficulties. If we call[22] the outer protoplasmic surface X and the inner surface Y we may say that although the X and Y layers of the protoplasm are undoubtedly different it is hard to see how a species which can diffuse in one direction in either layer cannot likewise diffuse in the opposite direction in the same layer. In particular NH_4X, if it enters the sap, must have diffused through Y. It ought then to be able to diffuse back through Y when the direction of the gradient is reversed by exposing the cells to running normal sea water. It is, of course, possible that no appreciable amount of NH_4X can form at the sap-protoplasm interface because there is little or no HX there. This could happen if HX is not formed in Y. But in that case we should expect it to diffuse into Y and to the sap-Y interface rather rapidly. Of course, if HX were formed only at the X-sea water interface and if its partition coefficient were low it might be lost in great part to the sea water. In that case in order for there to be any appreciable diffusion of NH_4X through the protoplasm the partition coefficient of the salt NH_4X would have to be much greater than that of the acid HX. This does not seem probable. Indeed we have supposed that HX, which is probably not one acid but the type member of a group of weak acids, is much more soluble in the non-aqueous protoplasmic surface than in aqueous solution.

To avoid the assumption that a one way permeability exists in any layer it might be assumed[23] that X is permeable to NH_4X but not to urea and Y is permeable to urea but not to NH_4X. On this basis the seat of formation and decomposition of both urea and NH_4X might be in the *W* layer, the aqueous layer in the protoplasm between the two non-aqueous layers *X* and *Y*. Let us suppose that urea is formed or decomposed at the sap-*Y* and *Y*-*W* interfaces, and NH_4X is formed or decomposed at the sea water-*X* and *X*-*W* interfaces. Under these conditions, when the cell is exposed to ammonia containing sea water, NH_4X is formed at the sea water-*X* interface and diffuses to *W*, where it is decomposed by CO_2 which is either formed at the *W*-*Y* interface or is diffusing from the sap-*Y* interface. If NH_4X cannot diffuse in *Y* it might accumulate as NH_4HCO_3 in *W* until $NH_{3_e} = NH_{3_w}$, but its entrance raises the pH of *W* and as a result the rate of formation of urea is increased as the enzyme activity increases. The urea diffuses through *Y* to the sap, but it cannot, we assume, diffuse much in *X* because of the low partition coefficient. The first urea entering the sap and decomposing there increases the concentration of undissociated ammonia and the pH is raised. This promotes the outward movement of KX for which there is a favorable partition coefficient in both *Y* and *X*. As a result the further decomposition of urea in the sap does not raise the pH since the loss of KX is equivalent to the loss[4] of KOH.

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[22] Osterhout, W. J. V., *Ergebn. Physiol.*, 1933, **35**, 967.

[23] Electrical evidence indicates that X and Y are quite different (*cf.* footnote 22).

One point remains to be cleared up. Since the pH of the sap is low when the urea first enters its rate of decomposition would be small so that we might expect an induction period in the entrance of ammonia. But we have never observed one. Two things contribute to make it unlikely that we should see one. First the amount of ammonia required to raise the pH is very small because of the poor buffer capacity of the sap, and second the reaction

$$2NH_3 + CO_2 \rightleftharpoons (NH_2)_2CO$$

is in equilibrium far over on the side of the reactants. Consequently even though the efficiency of the enzyme is low, it takes a comparatively short time to produce an increase in the pH of sap. Once this occurs the efficiency of the enzyme increases.

Let us now consider conditions at the steady state. We should expect the entrance of ammonia to cease as soon as concentrations of urea in sap and *W* are equal, which should be when the condition $(NH_3)^2_w(CO_2)_w = (NH_3)^2_{sap}(CO_2)_{sap}$ is fulfilled. But this cannot happen as long as NH_4X is entering W and is being decomposed by CO_2 to form NH_4HCO_3. This process, however, will cease when

$$(NH_3)_{\text{sea water}}(HX)_{epo} = (NH_3)_w(HX)_{epw}$$

where *epw* refers to the equilibrium layer in the X layer adjacent to W. But if $(HX)_{epo} = (HX)_{epw}$ which will be the case if HX is distributed equally throughout the X layer, the right hand terms in numerator and denominator cancel, and then at the steady state

$$(NH_3)_{s.w.} = (NH_3)_w$$

and a steady state throughout the system will occur when

$$(NH_3)^2_{s.w.}(CO_2)_w = (NH_3)^2_{sap}(CO_2)_{sap}$$

But if $(CO_2)_{sap} = (CO_2)_w$, which may well be the case, the steady state will occur when

$$(NH_3)^2_{s.w.} = (NH_3)^2_{sap}$$

or when

$$(NH_3)_{s.w.} = (NH_3)_{sap}$$

But from a previous paper[8] we derive the relationship that

$$(NH_3)_{eop}(HX)_{epo} = (NH_3)_{eip}(HX)_{epi}$$

at the steady state. In this expression *eop* and *eip* refer merely to particular layers of sea water and sap adjacent to the protoplasm, and for purposes of comparison of this expression with the one where *eop* and *eip* can be considered respectively equivalent to sea water and sap respectively. Further we can assume that corresponding activity coefficients in sap and sea water are equal and their concentrations may be substituted for activities.

Using data from the experiment described in the previous paper[8] we find that the steady state becomes = 0 when $[HX]_{epi} \div [HX]_{epo}$ is taken as equal to 6.7.

Now suppose we identify HX with CO_2. In order to satisfy the urea steady state equation $(CO_2)_{sap} \div (CO_2)_w$ will have to be equal[24]

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[24] Because for the steady state when urea is concerned,

$$\frac{[NH_3]^2_{s.w.}}{[NH_3]^2_{sap}} = \frac{[CO_2]_{sap}}{[CO_2]_{s.w.}} \qquad \begin{aligned}[NH_3]_{s.w.} &= 5.9 \times 10^{-5}\\ [NH_3]_{sap} &= 8.83 \times 10^{-6}\end{aligned}$$

to 45. This seems very unlikely in the case of CO_2 which, as we know from previous experiments,[25] diffuses very rapidly in the protoplasm. For this reason it seems unlikely that urea plays any part in the process of ammonia penetration if we regard HX as CO_2.[*]

Urea has served merely as an example of a possible species which might operate through an enzyme. Instead of forming urea with CO_2 ammonia might add on to an unsaturated linkage of an organic molecule elaborated by the protoplasm. And if one molecule of ammonia adds one molecule of the organic compound, we get for the two equations

$$(NH_3)_w(R)_w = (NH_3)_e(R)_w$$

and

$$\frac{(NH_3)_{s.w.}(HX)_{s.w.}}{(NH_3)_w(HX)_{s.w.}} = (NH_3)_w(HX)_{s.w.}$$

where R is an unsaturated molecule capable of adding on one amino group. If HX is distributed uniformly in the X layer of the protoplasm, we may combine the two equations to get

$$(NH_3)_{s.w}(R)_w = (NH_3)_{sap}(R)_{sap}$$

which is the same sort of equation as we obtained in the previous paper.

Before discussing possible reactions of the type suggested we shall consider the effects of light on the urea equilibrium.

It was observed that normal light increased both the rate of accumulation and the rate of exit of ammonia and that the induction period was shorter under normal light.

The increased rate of entrance in light is readily explained by assuming that due to photosynthesis the pH immediately at the sea water-X interface is raised so that the rate of diffusion of NH_4X through X is increased. This increases the rate of urea formation and therefore the rate of increase of the ammonia concentration in the sap. But the slowing up of the rates of exit and the lengthening of the induction period in the dark do not fit as well into the picture.

The ammonia emerging from the protoplasm is washed away by the flow of sea water to which the cells are exposed. Nevertheless there is a small but definite concentration of undissociated ammonia in the sea water and this should increase in the light at the sea water-protoplasm interface, so that the rate of diffusion of NH_4X through X should be decreased. The effect might be very small indeed because of the very low concentration of total ammonia in the sea water, but any effect would be in the opposite direction to that observed.

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[25] Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 695.

Light, however, may affect the rate of attainment of the urea equilibrium in other ways. It is not impossible that light alone affects the rate of urea synthesis and decomposition.[26] But it seems more likely that the results may arise from an increase in the activity of the enzyme.

Some recent results by Murakami[27] indicate that a number of enzymes, among them urease, are quite inactive in the dark, but become active when illuminated. However, the activity decreases as the intensity of the light increases. But Pincussen and Katô[28] found that prolonged exposure to ultraviolet light or sunlight gradually inactivates urease. Assuming, however, that moderate light increases the effectiveness of the enzyme in the cell we can see that this would explain all the observed effects. Especially it would explain why in the exit experiments the induction period is shorter in the light. For if the enzyme is more effective the rate of increase in the urea concentration in the sap will be greater, and the urea concentration at which the decrease of ammonia concentration in the sap begins to be appreciable will be attained sooner. Light might also influence the rate by increasing the amount of enzyme in the system. As is well known in general the rate at which equilibrium is attained in enzyme catalysis increases with the increase in the ratio of enzyme to substrate.

The question may be asked if it is necessary to assume a dual process for ammonia transport using both NH_4X and urea. There is the possibility that urea alone accounts for ammonia entrance and exit.

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[26] For photolysis of urea in sunlight see Rao, G. G., and Pandalai, K. M., *J. Indian Chem. Soc.*, 1934, **11**, 623. For the synthesis of urea from ammonium carbonate solution in ultraviolet light see Fearon, W. R., and M'Kenna, C. B., *Biochem. J.*, London, 1927, **21**, 1087.

[27] Murakami, R., *J. Agric. Chem. Soc.*, Japan, 1936, **12**, 151, 172, 180, 709; 1937, **13**, 46. These papers are in Japanese, but English abstracts are supplied.

[28] Pincussen, L., and Katô, N., *Biochem. Z.*, Berlin, 1923, **142**, 228

On this basis urea must be formed during entrance at the protoplasm-sea water interface in the presence of an enzyme. In the light several effects might be present: (*a*) the light might increase the effectiveness of the enzyme thereby speeding up the entrance of ammonia; (*b*) the light by inducing photosynthesis would raise the pH at the sea water-protoplasm interface and this might speed up entrance by bringing the system nearer the optimum pH for the enzyme; (*c*) the light might slow up the reaction by causing the photosynthetic removal of CO_2 and thus decreasing the concentration of one of the reactants needed for urea synthesis; or (*d*) the light might adjust the pH at a less favorable point for the enzyme, should the normal pH of sea water be nearer the optimum pH than that produced by photosynthesis. Thus light might have the net effect of increasing the speed greatly, leaving the speed unchanged, or decreasing it to greater or less extent.

If it is to increase the speed we should have to suppose that the enzyme effectiveness increases all the way from the pH of the sap about 5.6 up to 9.0–9.5 the probable pH in the layer of sea water adjacent to the protoplasm. This would be an unusually long range.

We now consider possible carriers other than urea, such as the amino acids, aspartic acid, $COOH(NH_2)CH \cdot CH_2COOH$; asparagine, $COOH(NH_2)CH\ CH_2CONH_2$; and glutamine, $COOH(NH_2)CH\ CH_2CH_2CONH_2$. All these are widely distributed in plants, particularly the last two which usually occur together.[29] All of these can lose ammonia in the presence of suitable enzymes. Aspartic acid can be deaminized readily in the presence of aspartase to fumaric acid. Thus,

$$l\text{-}COOH(NH_2)CH\ CH_2COOH \rightleftharpoons \underset{\text{fumaric acid}}{COOH\ CH = CHCOOH} + NH_3$$

This reaction, according to Jacobsohn and Tapadinhas[30] is a true reversible catalysis. The equilibrium is established according to results of Quastel and Woolf,[31] and Woolf[32] who used an enzyme preparation extracted from microorganisms when $K \dfrac{[NH_3]\ [\text{fumarate}]}{[l\text{-aspartate}]} = 0.01$. Borsook and Huffman[33] have calculated the free energy changes in the production of fumaric acid from *l*-aspartic acid, and have compared the result with that calculated from the equilibrium given by Quastel and Woolf. They agree with Jacobsohn and Tapadinhas that the reaction is a real reversible catalysis.

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[29] Schwab, G., *Planta*, 1936, **25**, 579.

[30] Jacobsohn, K. P., and Tapadinhas, J., *Biochem. Z.*, Berlin, 1935, **282**, 374.

[31] Quastel, J. H., and Woolf, B., *Biochem. J.*, London, 1926, **20**, 545.

[32] Woolf, B., *Biochem. J.*, London, 1929, **23**, 472.

[33] Borsook, H., and Huffman, H. M., *J. Biol. Chem.*, 1932, **99**, 663.

The optimum pH for the enzyme appears to be between 7.0 and 7.5,[34] and at 5.5 the action ceases. *l*-asparagine can be hydrolyzed in the presence of a specific enzyme, asparaginase, to *l*-aspartic acid, but it is not certain that this is a really reversible catalysis. Some investigators believe that the asparagine is completely hydrolyzed in the presence of the enzyme. Geddes and Hunter[35] support this view. They fix the optimum of the reaction about at pH 7.9 and find that it is still active down to 5.5 and up to 10.5. Suzuki[36] also found that the asparagine was completely hydrolyzed. His optimum value was 8.10. However, the results of Bach,[37] who was unable to get more than 80 per cent hydrolysis, suggest that an equilibrium is set up. Bach's optimum pH was 8.6 and the range of enzyme activity is from pH 6.5 to pH 10. Schmalfuss and Mothes[38] also believe that the reaction is a reversible one resulting in the formation of a salt, ammonium aspartate,

$$COOH\ CH(NH_2)CH_2CONH_2 + H_2O \rightleftharpoons COOH\ CH\ (NH_2)CH_2COONH_4$$

They fix the optimum at 7.7 − 7.8 and the range of activity of the enzyme from pH 6 to pH 10. It seems probable to us that the reaction is a reversible catalysis, but that the equilibrium point is far over on the side of the aspartic acid.

Whether or not this is so, it is clear that in the plant asparagine can be formed, possibly through the effect of another enzyme.[39]

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[34] According to Virtanen and Tarnanen (Virtanen, A. I., and Tarnanen, J., *Biochem. Z.*, Berlin, 1932, **250**, 193) the optimum is from 7.0 to 7.5 and the enzyme is no longer effective at 5.5.

[35] Geddes, W. F., and Hunter, A., *J. Biol. Chem.*, 1928, **77**, 197.

[36] Suzuki, Y., *J. Biochem.*, Japan, 1936, **23**, 57.

[37] Bach, D., *Bull. Soc. chim. biol.*, 1929, **11**, 119.

[38] Schmalfuss, K., and Mothes, K., *Biochem. Z.*, Berlin, 1930, **221**, 134.

[39] In this connection, Kultzscher (Kultzscher, M., *Planta*, 1932, **17**, 699) says that the equilibrium, amide nitrogen ⇌ ammonium salts, varies greatly with the pH of the sap. In plants with saps below pH 5, the tendency is to store nitrogen as ammonium salts and above that as amide. This suggests to us that in plants with saps of low pH the enzyme is so ineffective that amides such as asparagine and glutamine cannot form very rapidly. It should be noted that the NH_2 groups in aspartic and glutamic acids are amino groups not amide groups. An amino group is not hydrolyzed off in the presence of a hydrolase such as asparaginase. Indeed apparently it cannot be removed by hydrolysis which would leave an hydroxy acid, under natural conditions. But instead the amino group is oxidized off as ammonia, leaving behind an unsaturated acid.

The situation with glutamine is not yet clear. The recent work of Krebs[40] indicates that there are at least two enzymes which can be classed as glutamases. The one obtained from kidney tissue appears capable of hydrolyzing glutamine or causing its synthesis from glutamic acid and an ammonium salt. The optimum pH is at 7.4 and the range from 5.9 to 8.6 at least. The author did not explore the limits further.

Now if any one of these amino acids acts as ammonia carrier it may be expected to operate in the following way. If aspartic acid is concerned: In the presence of the enzyme fumaric acid acquires an amino group by uniting with undissociated ammonia thus setting up a gradient of aspartic acid across the protoplasm. The aspartic acid moves to the protoplasm-sap interface where it is decomposed in the presence of the enzyme and the undissociated ammonia goes into the sap. If this is the correct explanation accumulation could occur until $[NH_3]_i = [NH_3]_o$ provided the fumaric acid is distributed uniformly through the protoplasm if equilibrium could ever be established. In most cases we should not expect this. Instead, owing to the lower pH at the sap-protoplasm interface the enzyme would be less effective. Hence the concentration of aspartic acid at the sap-protoplasm interface would equal that at the sea water-protoplasm interface before $[NH_3]_i = [NH_3]_o$.

Either asparagine or glutamine would operate in the same way as aspartic acid except that the reaction in this case involves the loss of a molecule of water per molecule of ammonia reacting.

It is unnecessary to carry the argument further. The same considerations applying to the transport of ammonia as urea could apply to its transport as an amino group. But if ammonia enters by the formation of an amino group the scheme which best fits the experimental facts is as follows.

All the ammonia is transported as amino groups which add on to the unsaturated linkage of an organic species *R* elaborated wholly or chiefly at the sap-protoplasm interface.

This is preferable because the rate equation during accumulation which can be derived from it is analogous to the one found to fit in the previous paper except that in place of the acid *HX* we substitute the organic species *R*.

In a previous paper[3] the rate equation could be put in the form

$$p''' = \frac{2.3}{t} \log \frac{\frac{b}{c}a}{\frac{b}{c}a - x}$$

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[40] Krebs, H. A., *Biochem. J.*, London, 1935, **29**, 1951.

where P''' is the permeability constant, b = concentration of HX or alternately of R at the sea water-protoplasm interface, c = concentration of HX or R at the sap-protoplasm interface, and a and x are the concentration of undissociated ammonia in sea water and sap respectively. In the previous paper we interpreted the results to mean that

$$[HX]_{epi} = 6.7[HX]_{epo}$$

In the present case we assume that the effective concentration of $R_{epi} = R_{epo}$. The necessity for introducing R in place of HX is apparent only when we consider the exit of ammonia. The neutralization reaction to form NH_4X must be regarded as practically instantaneous, but the amination of the R may be slow and the induction period can be attributed to the relative ineffectiveness of the enzyme as a catalyst under certain conditions.

SUMMARY

The exit of accumulated ammonia from the sap of *Valonia macrophysa*, Kütz., into normal (nearly ammonia-free) sea water, has been studied in light (alternation of daylight and darkness) and in darkness. Exit is always preceded by an induction period lasting 1 or more days. This is longer in darkness. After exit starts the rate is greater in light than in darkness.

The pH of the sap drops off soon after the cells are exposed to normal sea water even before any definite decrease in the ammonia concentration of the sap has occurred. This suggests that the decrease in the pH is due to the loss of a very small amount of NH_3 or NH_4OH without a corresponding gain of sodium as a base.

In most cases sodium replaced the ammonia lost during exit, but there is some evidence that potassium may also replace ammonia.

To account for the induction period it is suggested that other species than NH_4X are concerned in the transport of ammonia, for example urea or amino acids.

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FORMATION OF TRYPSIN FROM CRYSTALLINE TRYPSINOGEN BY MEANS OF ENTEROKINASE

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The mechanism of formation of trypsin from trypsinogen by means of enterokinase has been a matter of almost continuous controversy since the discovery of enterokinase by Schepowalnikow in Pawlow's laboratory in 1899. Pawlow and Schepowalnikow considered enterokinase to be a typical enzyme. Studies of the kinetics of activation have shown, however, that while the rate of activation of a definite concentration of trypsinogen was proportional to the concentration of enterokinase used, the ultimate amount of trypsin formed was not independent of the concentration of enterokinase, as would be expected were enterokinase a true enzyme. This led to the suggestion that the formation of trypsin from trypsinogen by enterokinase is essentially a stoichiometric combination between trypsinogen and enterokinase to form an active enzyme, "trypsin-kinase" (1).

The isolation (8) of crystalline trypsinogen from fresh beef pancreas, its autocatalytic transformation at pH 7.0–9.0 into active trypsin without the aid of any outside activator, and the isolation of the active trypsin in pure crystalline form offer proof against the assumption that trypsin is a stoichiometric compound of kinase and trypsinogen. It was also found that pure crystalline trypsinogen can be changed into active trypsin at pH 3.0–4.0 by means of a kinase obtained from a mold of the genus *Penicillium* (2). The trypsin formed was crystallized and found to be identical in crystalline form, solubility, and specific activity with the crystalline trypsin obtained by spontaneous autocatalytic activation of trypsinogen at pH 8.0. The action of mold kinase was that of a typical enzyme, the process of activation following the course of a catalytic unimolecular reaction and the ultimate amount of trypsin formed being independent of the concentration of mold kinase used.

This paper deals with the kinetics of the formation of trypsin from crystalline trypsinogen by means of purified enterokinase obtained from swine duodenum contents. Enterokinase acts best in the range of pH 6.0–9.0 where autocatalytic formation of trypsin from trypsinogen occurs readily. The percentage rate of this reaction, however, is proportional to the concentration of trypsinogen. Hence, by using very dilute trypsinogen solutions the rate of the autocatalytic activation may be made negligible compared with that of the activation brought about by a significant amount of enterokinase (3). The autolysis of the trypsin formed, which generally occurs in the range of pH 7.0–9.0 (4), is also minimized by using dilute trypsinogen and by employing temperatures not higher than 5°C.

A further complication exists at pH 7.0–9.0 since under these conditions trypsinogen in the presence of trypsin is partly changed to an inert protein (5) which can no longer be transformed into trypsin either by enterokinase or mold kinase. This complication is minimized in solutions more acid than pH 6.0 where the rate of transformation of trypsinogen into inert protein is greatly reduced. When activation by enterokinase is allowed to proceed at pH below 6.0 enterokinase acts almost like a typical enzyme. The reaction follows approximately the course of a theoretical unimolecular reaction with a velocity constant proportional to the concentration of enterokinase used and the ultimate amount of trypsin formed is practically independent of the concentration of kinase.

If, on the other hand, the activation is allowed to proceed at pH above 6.0 a great portion of the trypsinogen is transformed into inert protein, the more so the lower the concentration of enterokinase used. As a result, the ultimate amount of trypsin formed is less as the concentration of enterokinase used is decreased in agreement with the findings of earlier workers (6).

The kinetics of the formation of the trypsin from crystalline trypsinogen by means of enterokinase under conditions where part of the trypsinogen is changed into inert protein can be derived mathematically as follows:

Let G_0 = initial concentration of trypsinogen
E = concentration of enterokinase
A = concentration of trypsin at any time t

A_e = final concentration of trypsin
I = concentration of inert protein formed from trypsinogen in any time t
I_e = final concentration of inert protein
$G_0 - A - I$ = concentration of trypsinogen at any time t.

Assuming (1) that the rate of formation of trypsin is proportional to the concentration of enterokinase and to the concentration of trypsinogen and, (2) that the rate of formation of inert protein is proportional to the concentration of trypsin and to the concentration of trypsinogen, we have the following equations:

$$\frac{dA}{dt} = K_1E(G_0 - A - I) \tag{1}$$

$$\frac{dI}{dt} = K_2A(G_0 - A - I) \tag{2}$$

Hence

$$\frac{dI}{dA} = \frac{K_2A}{K_1E} \tag{3}$$

and

$$I = bA^2 \tag{4}$$

where $b = \frac{K_2}{2K_1E}$ and K_1 and K_2 are the velocity constants of the reactions.

Substituting bA^2 for I in Equation 1, we get

$$\frac{dA}{dt} = K_1E(G_0 - A - bA^2) \tag{5}$$

At the end of the reaction when $\frac{dA}{dt} = 0$ we have

$$G_0 = A_e + bA^2_e \tag{6}$$

or

$$\frac{G_0 - A_e}{A^2_e} = b = \frac{K_2}{2K_1E} \tag{7}$$

Substituting $A_e + bA^2_e$ for G_0 in Equation 5 we get

$$\frac{dA}{dt} = K_0E\,[(A_e - A)(1 + bA_e + bA)] \tag{8}$$

which on integration gives

$$\ln\frac{A_e}{A_e - A} + \ln\left(1 + \frac{bA}{1 + bA_e}\right) = mt \tag{9}$$

where

$$m = K_0E + K_2A_e \tag{10}$$

The exponential form of Equation 9 is

$$A = \frac{A_e(e^{mt} - 1)}{e^{mt} + \dfrac{bA_e}{1 + bA_e}} \tag{11}$$

where m is the slope of the straight line obtained when the values for

$$\ln\frac{A_e}{A_e - A} + \ln\left(1 + \frac{bA}{1 + bA_e}\right)$$

are plotted against t, in accordance with Equation 9.

It follows from Equations 7 and 10 that

$$K_0E = \frac{mA_e}{2G_0 - A_e} \tag{12}$$

and

$$K_2 = \frac{m - K_0E}{A_e} \tag{13}$$

At pH more acid than 6.0 K_2 becomes negligible and Equations 9 and 11 are then reduced to the approximate forms

$$\ln\frac{G_0}{G_0 - A} = K_0Et \tag{9a}$$

and

$$A = G_0(1 - e^{-K_0Et}) \tag{9b}$$

which are the equations of a simple catalytic unimolecular reaction. If an appreciable amount of trypsin, A_0, is present as an impurity in the sample of trypsinogen used then Equation 9 becomes

$$\ln\frac{A_e - A_0}{A_e - A} + \ln\frac{1 + bA_e + bA}{1 + bA_e + bA_0} = mt$$

where

$$b = \frac{G_0 + A_0 - A_e}{A^2_e - A^2_0}$$

also Equation 4 becomes

$$I = b(A^2 - A^2_0)$$

The derived equations bring out the following relationship between the trypsin and inert protein formed from trypsinogen in the presence of enterokinase at pH above 6.0: the concentration of inert protein formed at any time during the reaction is proportional to the square of the concentration of trypsin formed and inversely proportional to the concentration of enterokinase used (Equation 4).

It follows then that the higher the concentration of enterokinase used the greater is the percentage of trypsinogen changed into active trypsin.

This relationship, as well as the equation for the kinetics of the enterokinase activation, has been found to check closely with the experimental results.

The transformation of trypsinogen into trypsin in the presence of enterokinase appears thus to be a typical enzyme reaction catalyzed by the enzyme enterokinase. The anomalous results found under certain conditions are due to a secondary reaction by which trypsin changes trypsinogen to an inert protein.

The kinetics of the reaction outlined above applies only to dilute solutions of purified trypsinogen. The activation of concentrated solutions of trypsinogen is complicated by rapid autocatalytic formation of trypsin by the trypsin itself. The activation of crude pancreatic extracts is much more complicated since, as previously noted (7), these extracts contain chymo-trypsinogen in addition to trypsinogen and also a substance which inhibits trypsin (8). In outline the activation of crude trypsinogen by enterokinase proceeds as follows: Addition of kinase transforms the trypsinogen to trypsin which catalyzes the conversion of trypsinogen to form more trypsin and which also catalyzes the conversion of chymo-trypsinogen to chymo-trypsin. If the method of activity determinations used determines both trypsin and chymo-trypsin, as is usually the case, the curves obtained when the activity of the solution is plotted against time are S shaped but asymmetrical and resemble those obtained by Vernon (9). These curves generally show a long initial lag period which is partly caused by the interference of the trypsin inhibitor with the catalytic action of the trypsin formed.

Experimental Studies of the Kinetics of the Formation of Trypsin from Crystalline Trypsinogen by Means of Enterokinase. General Procedure

Reaction mixtures were made up of solutions of crystalline trypsinogen and of enterokinase in dilute buffers and allowed to stand at 5°C. The solutions were kept sterile by the addition of 0.1 ml. 1 per cent merthiolate in 1.4 per cent borax solution to 100 ml. of reaction mixture. Samples of 1 ml. were acidified with hydrochloric acid to about pH 2.0 in order to stop the reactions. The concentration of trypsin in the samples was then determined by the hemoglobin method of Anson (10). Samples were also taken in some cases for the determination of the concentration of inert protein formed during the reaction by the method described elsewhere (11) and which consists essentially in adding to the samples a large excess of enterokinase and thus bringing about rapid and complete activation of all the available trypsinogen.

Kinetics of Formation of Trypsin by Means of Enterokinase at pH 5.6 and 7.6.—The striking difference in the behavior of enterokinase when allowed to act on crystalline trypsinogen at pH 5.6, as compared with that of pH 7.6, is shown in Fig. 1. At pH 5.6 enterokinase acts almost like a typical enzyme so that the ultimate concentration of trypsin formed in a solution of trypsinogen of a definite concentration varies only slightly with the concentration of enterokinase used, while at pH 7.6 the ultimate concentration of trypsin formed varies markedly with the concentration of enterokinase.

Fig. 2 shows the experimental data for the action of enterokinase on crystalline trypsinogen at pH 5.6 plotted logarithmically (Equation 9*a*).

$$\ln \frac{G_0}{G_0 - A} \textit{ vs.}$$

In the calculations the value of G_0 was taken as 1.5×10^{-3} $[\text{T. U.}]^{\text{Hb}}$ per ml. which is the value obtained in the presence of a large excess of enterokinase. In the presence of the concentrations of kinase used in this experiment (Fig. 1, pH 5.6) the activity reaches a maximum value of only $1.3\text{–}1.35 \times 10^{-3}$ $[\text{T. U.}]^{\text{Hb}}$ per ml., the difference being due to the formation of a small amount (10–20 per cent) of inert protein. In the first part of the reaction this formation of inert

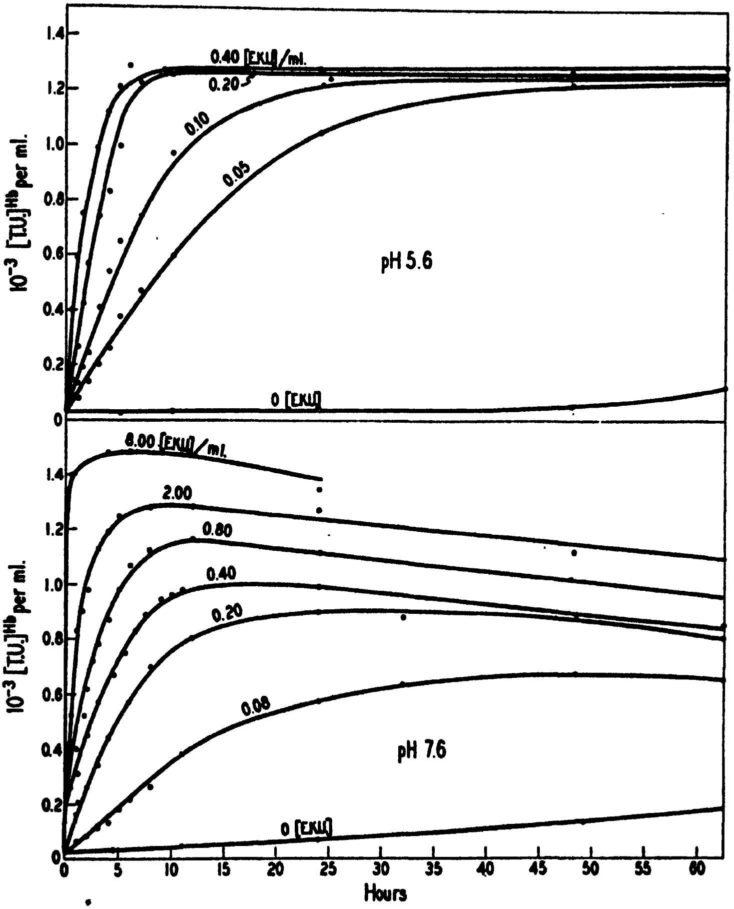

FIG. 1. Formation of trypsin from crystalline trypsinogen by enterokinase at 5°C. Activation mixtures: 5 ml. 0.065 per cent solution of trypsinogen in 0.005 M hydrochloric acid + 10 ml. 0.1 M phosphate buffer + 1.0 ml. enterokinase solution in water + distilled water to 50 ml.

protein is too small to affect the results but in the latter part of the reaction when A approaches the value of G_0 the reaction will proceed more slowly than calculated on the simple assumption that no inert protein is formed. Thus in Fig. 2, where the results have been plotted by the simple monomolecular equation the theoretical and experimental points agree up to 50–60 per cent activation and the slopes of the lines are proportional to the concentrations of enterokinase used. In the last 30–40 per cent of the reaction the formation of inert protein becomes significant and the experimental points lie below those predicted by the simple equation in which the formation of inert protein was neglected.

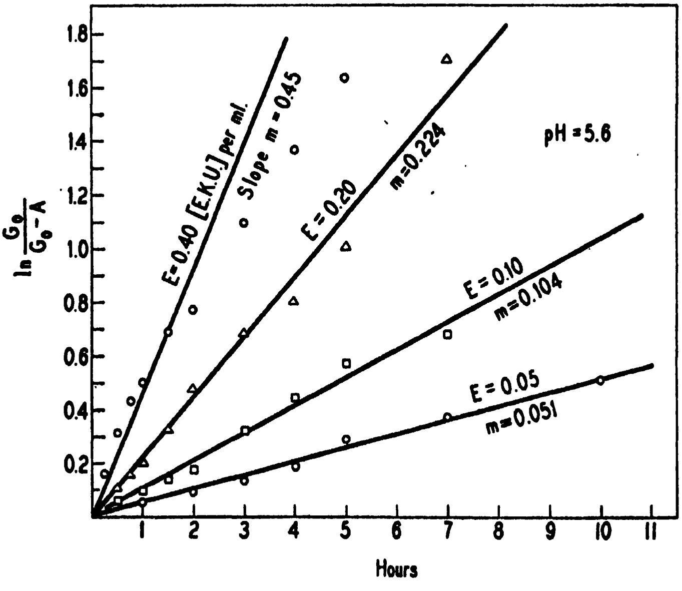

FIG. 2. Logarithmic curves of Fig. 1 pH 5.6 plotted in accordance with the equation of a simple unimolecular reaction.

On the other hand, curve I in Fig. 3 shows that the experimental data for the action of enterokinase at pH 7.6 do not fall in straight lines when plotted logarithmically in accordance with the theoretical equation of a simple unimolecular reaction even in the first part of the reaction. The experimental points, however, do fall in a straight line (curve II, Fig. 3) when plotted according to the more complete Equation 9 which takes care of the complication due to the formation of inert protein. Curve III of Fig. 3 shows the close agreement between the observed and theoretical values of A. The last were computed by means of Equation 11 with $m = 0.21$ as given by the slope of curve II. The logarithmic curves (Equation 9) for the whole series of pH 7.6 are shown in Fig. 4. In practically all cases the experimental points lie on straight lines. The slopes of the various lines, as well as the calculated values of K_e, K_2, and A_e are given in Table I. The concentrations of G_0 and A_e are expressed in mg. protein nitrogen per ml., 1 mg. protein nitrogen being equivalent to 0.15 [T. U.]$^{\text{Hb}}$.

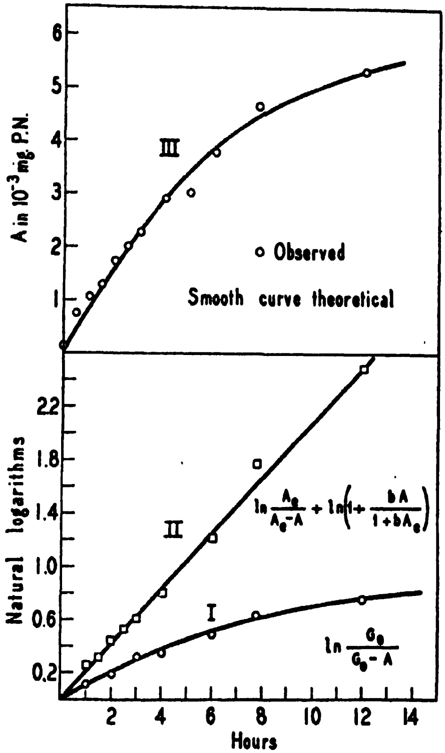

FIG. 3. Comparison between theoretical and observed data on the formation of trypsin from crystalline trypsinogen by enterokinase at pH 7.6 and 5°C. Concentration of trypsinogen 0.01 mg. protein nitrogen per ml. Concentration of enterokinase 0.20 [E.K.U.] per ml.

Formation of Inert Protein.—Fig. 5 shows the time curves for the simultaneous formation of inert protein and of trypsin from trypsinogen in the presence of enterokinase at pH 7.6 both determined in-

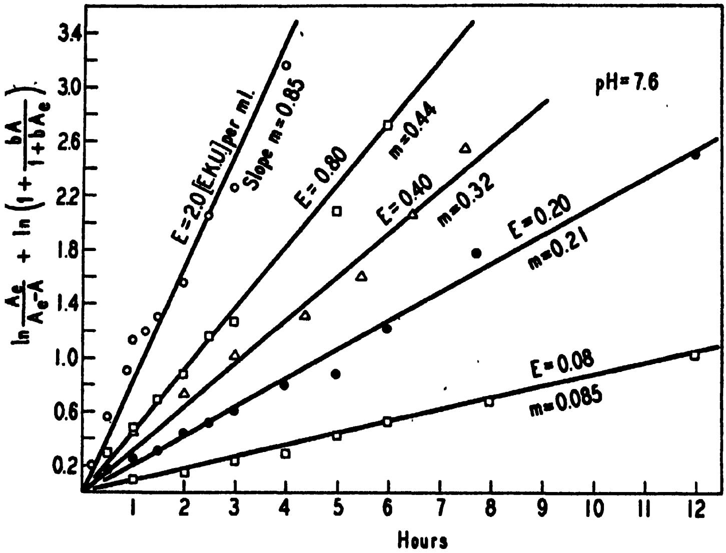

FIG. 4. Logarithmic curves of Fig. 1 pH 7.6 plotted in accordance with Equation 9.

TABLE I

Effect of Concentration of Enterokinase on the Formation of Trypsin at pH 7.6 and 5°C.

Trypsinogen concentration G_0 = 0.01 mg. protein nitrogen per ml.

Concentration of enterokinase in [E.K.U.] per ml..................................	0.08	0.20	0.4	0.8	2.0
A_e in mg. trypsin protein nitrogen per ml....	0.0045	0.0060	0.0066	0.0077	0.0085
Slopes $m = K_eE + K_2A_e$..................	0.085	0.21	0.32	0.44	0.85
K_e (Equation 12) per [E.K.U.] per hr.......	0.31	0.45	0.40	0.35	0.31
K_2 (Equation 13) per mg. trypsin protein nitrogen per hr.........................	14	20	25	21	27

dependently. The plotted experimental points fall closely on the smooth theoretical curves. The theoretical values of A were obtained by means of Equation 11. The theoretical values of I were calculated from the theoretical values of A by means of Equations 4 and 7, namely

$$I = bA^2$$

where

$$b = \frac{G_e - A_e}{A^2_e} = \frac{(10 - 5) \times 10^{-3}}{25 \times 10^{-6}} = 200$$

The relation between the concentration of trypsin and inert protein formed in the reaction mixture at any time is shown graphically in

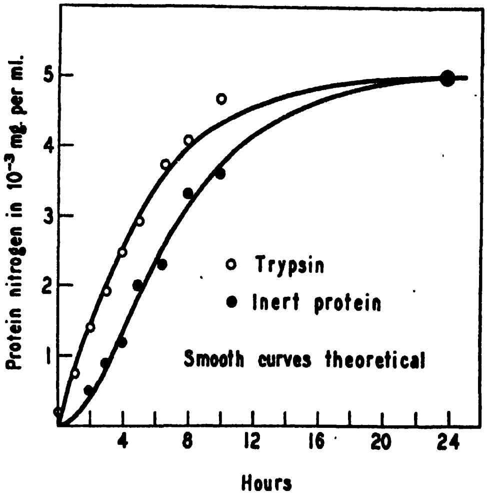

FIG. 5. Comparison between theoretical and observed data on formation of trypsin and inert protein from crystalline trypsinogen pH 7.6 and 5°C. in the presence of enterokinase. Concentration of trypsinogen 0.01 mg. protein nitrogen per ml. Concentration of enterokinase 0.20 [E.K.U.] per ml. in 0.02 M phosphate buffer pH 7.6.

Fig. 6 where the values of I were plotted as a function of A. The smooth parabolic curve is the locus of the theoretical function $I = 200\ A^2$.

It should be observed that in the case of autocatalytic formation of trypsin described elsewhere the relation between I and A is linear (12), while here

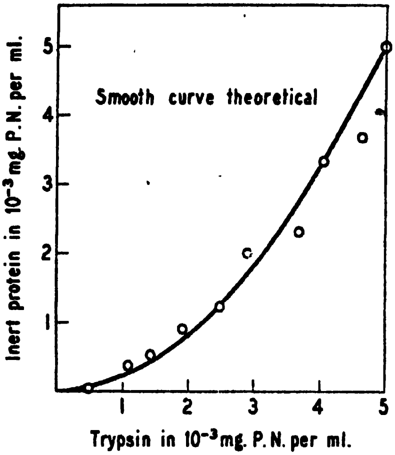

FIG. 6. Relation between trypsin and inert protein formed from crystalline trypsinogen at pH 7.6 and 5°C. in the presence of enterokinase. The smooth curve is the theoretical locus of the equation $I = bA^2$ where $b = \frac{G_e - A_e}{A_2} = 200$.

TABLE II

Effect of pH

Activation mixture: 1 ml. 1 M KH_2PO_4 + K_2HPO_4 mixtures, plus 1.0 ml. enterokinase, 5 [E.K.U.] per ml. water, plus 5.0 ml. crystalline trypsinogen, 0.1 mg. protein nitrogen per ml. M/200 hydrochloric acid, plus water to 50 ml. Samples 1.0 ml. plus 1.0 ml. 0.04 M hydrochloric acid for activity measurements.

Trypsinogen concentration G_e = 0.01 mg. protein nitrogen per ml.

Enterokinase concentration E = 0.1 [E.K.U.] per ml.

pH	5.26	5.78	6.12	6.38	6.55	6.72	6.94	7.18	7.45	7.75
A_e in 10^{-3} mg. trypsin protein nitrogen per ml	8.1	7.3	6.1	5.6	4.6	4.3	3.8	3.4	3.2	2.9
$K_eE + K_2A_e$ per hr. (from logarithmic plot Equation 9)	0.053	0.13	0.20	0.24	0.27	0.25	0.30	0.35	0.36	0.40
K_e per [E.K.U.] per hr. (Equation 12)	0.36	0.75	0.87	0.93	0.79	0.68	0.71	0.72	0.68	0.67
K_2 per mg. trypsin protein nitrogen per hr. (Equation 13)	2	8	19	26	41	43	60	82	93	113

in the presence of enterokinase I is proportional to the *square* of A. Both relations, however, are derived mathematically on the basis of the same assumption that the formation of inert protein is catalyzed by the trypsin formed.

Effect of pH on the Velocity Constants of Both Reactions

The striking difference in the kinetics of formation of trypsin by means of enterokinase at pH 5.8 and at pH 7.6 was shown in Fig. 1. A summary of a series of experiments on the effect of pH in the region of 5.26–7.75 on the kinetics of formation of trypsin from trypsinogen in the presence of enterokinase at 7°C. is given in Table II and also in Fig. 7.

The results show that there is a gradual decrease in the amount of trypsin formed with the increase of pH. At pH more acid than 5.0 there is, however, a sudden drop in the amount of trypsin formed due to destruction of enterokinase. The optimum range of pH for the rate of formation of trypsin by means of enterokinase is at about 6.2 as shown on curve K_e, Fig. 8. The values of K_2 for the various pH, as calculated from the slopes of the logarithmically plotted curves, are of the same magnitude as those obtained under the same approximate conditions of temperature, salt, and trypsinogen concentration in the absence of enterokinase during the autocatalytic formation of trypsin (13).

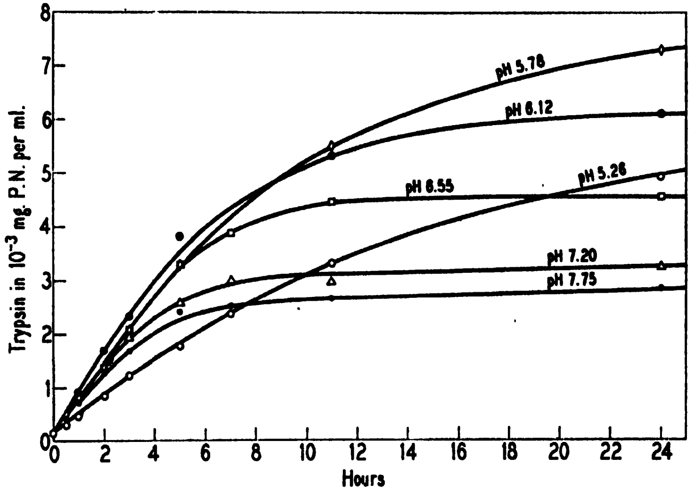

FIG. 7. Formation of trypsin at 7°C. by means of enterokinase at various pH. G_0 = 0.01 mg. trypsinogen protein nitrogen per ml. Enterokinase = 0.1 [E.K.U.] per ml.

Effect of Concentration of Trypsinogen

With increase in concentration of trypsinogen in solution, the concentration of enterokinase being kept constant, the rate of the catalytic formation of inert protein is greatly increased as compared with the rate of the catalytic formation of trypsin by enterokinase, since the relative rate of formation of the two products is proportional to the concentration of trypsin formed (Equation 3). The complicating effect of the formation of inert protein on the kinetics of the enterokinase action as the concentration of trypsinogen is increased becomes evident even at pH 5.8. This is shown in Fig. 9 where the percentage of trypsinogen changed into trypsin was plotted against t. The higher the concentration of trypsinogen used the lower was the percentage rate as well as the final per cent of trypsinogen changed into trypsin.

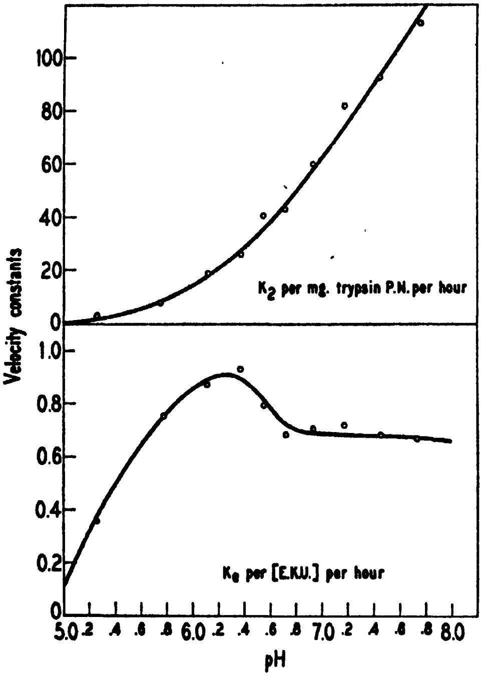

FIG. 8. Effect of pH on the velocity constants

The results of the experiment are summarized in Table III where the observed values of inert protein formed are given. In every case the sum of values of A_e and I_e is equal to the corresponding value of G_o.

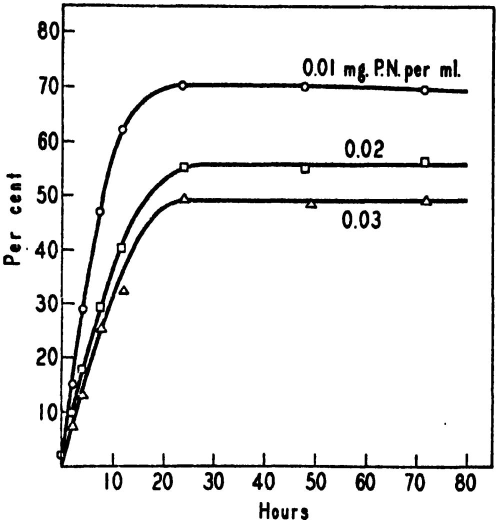

FIG. 9. Effect of concentration of trypsinogen on the formation of trypsin from crystalline trypsinogen by means of enterokinase at pH 5.8 and 6°C. Concentration of enterokinase 0.1 [E.K.U.] ml.

TABLE III

Effect of Concentration of Trypsinogen at pH 5.8 and 6°C.

Concentration of enterokinase 0.08 [E.K.U.] per ml. 0.02 M phosphate buffer pH 5.8 of activation mixture.

Concentration of trypsinogen = G_o, in mg. protein nitrogen per ml. activation mixture	0.01	0.02	0.03
Final concentration of trypsin formed = A_e, in trypsin mg. protein nitrogen per ml.	0.007	0.011	0.015
Final concentration of inert protein formed = I_e (observed) in mg. protein nitrogen per ml.	0.003	0.008	0.015
$\frac{K_2}{K_e} = \frac{2E(G_e - A_e)}{A_e^2}$	10	12	11

The table also shows that the relation between G_e and A_e in each case checks with the theoretical Equation 7 giving a value of $\frac{K_2}{K_e}$

independent of the original concentration of trypsinogen used. The values of K_e and K_2 as calculated from the slopes of the logarithmic curves appear, however, to decrease with increase in concentration of trypsinogen. A similar effect of increase in concentration of substrate on the magnitude of the velocity constant has been frequently observed in the case of enzymatic reactions (14).

Addition of fresh trypsinogen to the activation mixtures at the end of the reaction always brought about formation of more trypsin, thus proving that the incomplete activation of the original trypsinogen was not due to any insufficiency or possible inactivation of the enterokinase. On the other hand, the addition of excess enterokinase or mold kinase to the activation mixture at the end of the reaction has never brought about an increase in the concentration of trypsin although no significant loss of protein has been noticed. It is evident that the incompleteness of the enterokinase reaction is due to the partial transformation of the trypsinogen into inert protein which cannot be changed into trypsin by any known activator.

Methods

1. Preparation of Crystalline Trypsinogen.—The trypsinogen was prepared by the method of Kunitz and Northrop (8). The crystals were purified and made inhibitor free by means of trichloracetic acid as described on page 993 of the same reference, except for an extra step in the process which was omitted through a typographical error. The corrected procedure for purification by means of trichloracetic acid is as follows: 10 gm. filter cake of trypsinogen crystals is dissolved in 200 ml. N/400 hydrochloric acid and 200 ml. 5 per cent trichloracetic acid added. The solution is left at 20°C. for 1 hour and then filtered with suction and washed several times with small amounts of 2.5 per cent trichloracetic acid and finally with water. The semi-dry precipitate is dissolved in 25 times its weight of N/50 hydrochloric acid, allowed to stand about 30 minutes. Ammonium sulfate is added to 0.4 saturation. The precipitate is filtered off and rejected. The filtrate is brought to 0.7 saturation with solid ammonium sulfate and filtered with suction. The filter cake is dissolved in 3–5 times its weight of N/200 hydrochloric acid and dialyzed for 24 hours at 5–6°C. against running N/200 hydrochloric acid.

2. Preparation of Enterokinase.—A stock of enterokinase in water and containing about 1,000 kinase units per ml. was prepared from intestinal contents by the method described elsewhere (15).

3. Estimation of Enterokinase.—The quantity of enterokinase in any solution is expressed in terms of the velocity with which it transforms crystalline trypsinogen into trypsin under standard conditions. One enterokinase unit, 1 [E.K.U.] is the amount of kinase that brings about the activation of 0.065 mg. crystalline trypsinogen (0.01 mg. protein nitrogen) in 0.02 M Sørensen's phosphate buffer pH 5.8 at the rate of 100 per cent per hour at 5°C. Under these conditions the activation by enterokinase follows approximately the course of a simple unimolecular reaction so that the plotted values of $\ln \frac{G_0}{G_0 - A}$ *vs.* t fall in straight lines (Fig. 2) the slopes of which are proportional to the concentration of enterokinase used. The concentration of enterokinase in each case can be taken as equal to the slope of the lines by assigning the value of unity to the proportionality constant.

The standard method of estimating kinase involves the determination of concentration of trypsin in a series of samples taken at various intervals of time from the activation mixture in order to obtain several points for the logarithmic curve. For practical purposes the following simplified procedure was adopted: *Activation mixture:* 3 ml. 0.02 M phosphate buffer pH 7.6 plus 1.0 ml. enterokinase in 0.02 M phosphate pH 7.6 plus 1 ml. standard crystalline trypsinogen solution (0.1 mg. protein nitrogen per ml.) in N/200 hydrochloric acid. The activation mixture is placed for 30 minutes in a water bath at 25°C. 1.0 ml. of the mixture is then added to 5.0 ml. Anson's urea-hemoglobin solution; its trypsin content $[\text{T.U.}]^{\text{Hb}}$ is determined as described by Anson (10). The concentration of enterokinase in [E.K.U.] per ml. activation mixture corresponding to the $[\text{T.U.}]^{\text{Hb}}$ measured is then read off a standard curve. The standard curve is obtained by plotting the data of $[\text{T.U.}]^{\text{Hb}}$ *vs.* [E.K.U.] for a series of activation mixtures containing various dilutions of a stock of enterokinase of known [E.K.U.] content, as determined by the standard method. 1 [E.K.U.] is equivalent to about 100 mg. of acetone dried pigs' duodenal mucosa.

4. *Estimation of Trypsin.*—Method of Anson (10).

5. *Estimation of Inert Protein.*—Described in preceding paper (11).

The writer was assisted by Margaret R. McDonald.

SUMMARY

Crystalline trypsinogen is most readily and completely transformed into trypsin by means of enterokinase in the range of pH 5.2–6.0 at 5°C. and at a concentration of trypsinogen of not more than 0.1 mg. per ml. The action of enterokinase under these conditions is that of a typical enzyme. The process follows closely the course of a catalytic unimolecular reaction, the rate of formation of trypsin being proportional to the concentration of enterokinase added and the ultimate amount of trypsin formed being independent of the concentration of enterokinase.

The catalytic action of enterokinase on crystalline trypsinogen in

dilute solution at pH more alkaline than 6.0 and in concentrated solution at pH even slightly below 6.0 is complicated by the partial transformation of the trypsinogen into inert protein which can no longer be changed into trypsin even by a large excess of enterokinase. This secondary reaction is catalyzed by the trypsin formed and the rate of the reaction is proportional to the concentration of trypsin as well as to the concentration of trypsinogen in solution. Hence under these conditions only a small part of the trypsinogen is changed by enterokinase into trypsin while a considerable part of the trypsinogen is transformed into inert protein, the more so the lower the concentration of enterokinase used.

The kinetics of the formation of trypsin by means of enterokinase when accompanied by the formation of inert protein can be explained quantitatively on the theoretical assumption that both reactions are of the simple catalytic unimolecular type, the catalyst being enterokinase in the first reaction and trypsin in the second reaction.

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PURIFICATION AND CONCENTRATION OF ENTEROKINASE

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Waldschmidt-Leitz (1) has described a method of purification of kinase which consists essentially in drying swine duodenal mucosae with acetone and ether, extracting the dried mucosae with dilute ammonium hydroxide, and removing foreign protein from the aqueous extract by means of dilute acetic acid. The solution can be further purified by precipitation with alcohol, tannic acid, and finally by fractional adsorption on Al (OH)$_3$ and kaolin. By this method Waldschmidt-Leitz obtained a preparation which was about 100 times more active per unit dry weight than the acetone dried mucosa.

This paper describes a simplified method of purification of enterokinase whereby preparations of enterokinase 5,000–50,000 times as active as the acetone dried mucosa can be readily obtained.

The fluid contents of duodena of swine were found to be the most convenient source for enterokinase. The purification consisted principally in pH adjustment and fractional precipitation with ammonium sulfate. The contents of duodena of a large number of freshly killed swine were collected in the slaughter house and stored at about −10°C. The material was afterwards worked up gradually in the laboratory. It has been found that storage of the material frozen as long as a month did not affect its quality as a source of enterokinase.

The details of the process for purification and concentration of the enterokinase are as follows:

1. 2.5 liters of duodenal contents are diluted with 7.5 liters of tap water and warmed to 20°C.

2. 50 gm. of "Hyflo Super-Cel"[1] are mixed with every liter of mix-

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[1] Manufactured by Johns-Manville Corporation, 22 East 40th Street, New York City.

ture and the whole mass filtered with suction through filter cloth in a 15 inch Buchner funnel. First extract.

3. Residue is resuspended in 3 liters of tap water and refiltered through cloth.

4. Combined extracts are cooled to 5°C.

5. pH of extracts is adjusted with 5 N sulfuric acid to about 4.0 (tested with methyl orange). The precipitate formed is filtered rapidly with suction with the aid of 20 gm. of "Standard Super-Cel" per liter of solution.

6. Filtrate is brought immediately to pH 8.0 with 5 N sodium hydroxide.

TABLE I

Enterokinase from 2.5 Liters of Duodenal Contents

	Total enterokinase units [E.K.U.]	Per cent	[E.K.U.] per mg. protein nitrogen	[E.K.U.] per mg. carbohydrate	[E.K.U.] per mg. dry weight
First extract	194,000		100	16	
Washings	69,000		124	20	
Total	263,000	100			
pH 4.0 filtrate	200,000	76	275	19	
Filtrate from 0.4 saturated ammonium sulfate	163,000	62	760	675	40
Acetone dried pig mucosa					0.01

7. Solid ammonium sulfate is added to bring the filtrate to 0.8 saturation. The pH of the solution is again adjusted to pH 8.0 with 5 N sodium hydroxide. 4.0 ml. of 0.4 M pH 9.0 borate buffer is then added to every liter of solution. The formed flocculent precipitate is allowed to rise to the surface and is then easily collected into a dough-like mass and removed from the solution. Weight of precipitate about 20 gm.

8. The precipitate is dissolved in about 5 volumes of cold water and solid ammonium sulfate is added to 0.4 saturation. Filtered with suction with the aid of 5 per cent "Standard Super-Cel." Residue rejected.

9. Filtrate from 0.4 saturated ammonium sulfate is brought to 0.8 saturation with solid ammonium sulfate and filtered with suction. Filter cake about 15 gm.

10. Steps 8 and 9 repeated.

The degree of purity of the products obtained in the various steps as well as the yields are given in Table I.

Table I shows that one fractionation with ammonium sulfate raises the kinase activity of the material by 200 per cent with respect to protein and by 3,000 per cent with respect to carbohydrate.

The kinase content per milligram dry weight of the material is about 4,000 times as great as that of acetone dried duodenal mucosa.

The material can be further purified by dialysis and by repeated fractionation with ammonium sulfate as well as by fractional precipitation in cold 60 per cent alcohol or cold 2.5 per cent trichloracetic acid. It was found, however, that the material obtained after the first fractionation with saturated ammonium sulfate was free of any impurities likely to interfere with its use as an activator of trypsinogen.

TABLE II

Approximate Composition of Purified Enterokinase

	Per cent
Carbon	45
Total nitrogen	12
Protein precipitable in 2.5 per cent trichloracetic acid	10 or less
Carbohydrate	10 " "
Glucosamine	10 " "

The filter cake from 0.8 saturated ammonium sulfate is completely soluble in water and thus a solution of any desired strength is readily available.

An approximate analysis of the material twice refractionated with ammonium sulfate and then dialyzed is given in Table II. The relative content of protein, carbohydrate, and glucosamine varied considerably in different preparations and no definite conclusion can be drawn as yet as to the actual chemical nature of enterokinase.

Methods

1. Estimation of Enterokinase Activity.—The concentration of enterokinase is expressed in terms of enterokinase units [E.K.U.] per ml. One [E.K.U.] is equivalent approximately to the activity contained in about 100 mg. of acetone dried pigs' duodenal mucosa. The details of procedure of estimation, as well as the definition of the enterokinase unit used are described in the preceding paper (2).

2. *Protein Nitrogen.*—The protein nitrogen was determined by the turbidity method (3).

3. *Total Carbohydrate.*—Total carbohydrate was determined colorimetrically by the orcinol method of Sørensen and Haugaard (4) and expressed in terms of milligrams of sucrose.

4. *Glucosamine.*—Adaptation of the colorimetric method of Elson and Morgan (5). The procedure adopted for measuring the glucosamine content of the material used here is as follows: 1 ml. sample is mixed in a Pyrex 12 × 1.5 cm. tube, marked to 10 ml., with 1 ml. 2 M hydrochloric acid and heated for 1 hour at 100°C. in a steam bath under a reflux condenser about 25 cm. long and 6 mm. in diameter.[2] The solution is cooled, then the following reagents are added: 1 drop 0.1 per cent phenolphthalein, 1.0 ml. 2 M sodium hydroxide, a few drops of M/1 sodium carbonate to pink color, 1.0 ml. of freshly prepared solution of acetyl acetone (0.2 ml. in 10 ml. M/1 sodium carbonate). The mixture is reheated for 15 minutes at 100°C. under the reflux condenser, then cooled: 4.0 ml. 95 per cent alcohol is added and mixed. 1.0 ml. of Ehrlich's reagent (0.8 gm. *p*-dimethylaminobenzaldehyde in 30 ml. 95 per cent alcohol plus 30 ml. concentrated hydrochloric acid), and then alcohol to the 10 ml. mark are added. The solution is carefully but thoroughly mixed and filtered through 9 cm. Whatman's No. 42 filter paper in order to remove CO_2 bubbles as well as any turbidity from the liquid. The color of the solution is compared after 15 minutes with that of a standard glucosamine hydrochloride solution treated in the same manner.

The writer was assisted in this work by Margaret R. McDonald and Vivian Kaufman.

SUMMARY

A concentrated solution of purified enterokinase is conveniently prepared from the fluid contents of pigs' duodena by means of fractional precipitation with ammonium sulfate under the proper pH conditions.

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[2] Digestion in M/1 hydrochloric acid longer than 1 hour does not increase the value of the glucosamine readings in the case of this material. Other materials have to be digested longer or in more concentrated hydrochloric acid as found by Palmer, Smyth, and Meyer (6) and also by Sørensen (7).

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STUDIES ON ANTIBACTERIAL IMMUNITY INDUCED BY ARTIFICIAL ANTIGENS

I. IMMUNITY TO EXPERIMENTAL PNEUMOCOCCAL INFECTION WITH AN ANTIGEN CONTAINING CELLOBIURONIC ACID

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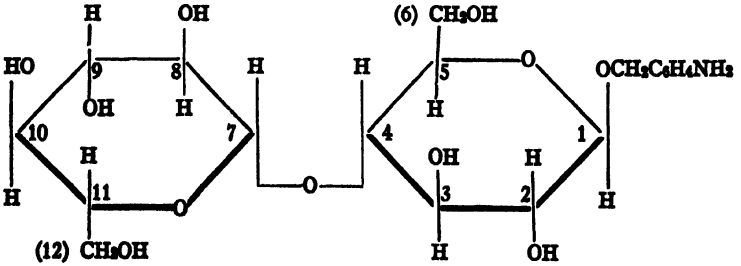
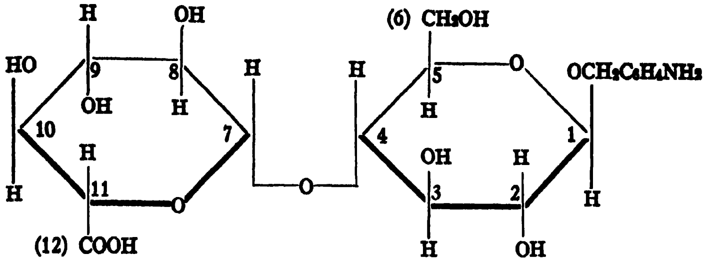
The results of experimental studies on artificial antigens containing the azobenzyl glycosides of glucuronic, galacturonic, and cellobiuronic acids indicate that the hexose uronic and aldobionic acids have an important function in determining the immunological characteristics of certain of the specific polysaccharides of encapsulated microorganisms (1). Azoproteins containing these uronic acids have the property of precipitating in high dilutions in antipneumococcal sera of various types, whereas antigens containing the azobenzyl glycosides of the corresponding aldoses show little or no serological activity. That the hexose uronic acid antigens actually combine with and precipitate the type specific polysaccharide antibodies has been demonstrated in a number of ways. It is apparent, therefore, that the artificial hexose uronic acid antigens possess certain of the serological characteristics of the immunologically active pneumococcus polysaccharides themselves. Despite this similarity, however, it has thus far been impossible to induce antibacterial immunity by injecting animals with the glucuronic or galacturonic acid antigens. Attempts to induce immunity to Type III pneumococcal infections in mice, rabbits, goats, and horses with an azoprotein containing glucuronic acid have all been unsuccessful. The reason probably resides in the fact that glucuronic acid alone does not approximate closely enough the chemical structure of the more complex building stone of the Type III pneumococcus polysaccharide, cellobiuronic acid (2).

Recently it has been shown that an azoprotein containing cellobiuronic acid simulates much more closely the serological characteristics of the capsular polysaccharide of Type III Pneumococcus than does one containing glucuronic acid (1 *c*). These observations have led to the belief that in order to induce Type III antipneumococcal immunity in experimental animals with an artificial antigen, it is not necessarily essential to have as the immuno-specific group the long chained type specific polysaccharide (3), but that the building stone from which it is constituted, namely the aldobionic acid, should suffice. In accepting this point of view it must be borne in mind that the polysaccharides, biologically active and otherwise, are simpler entities than are the antigenic proteins. The carbohydrates may in general be regarded as constituted from a fundamental pattern unit of one or more simple saccharides combined in glycosidic union to form a long chained macromolecule. Whether in artificially compounded antigens the integrity of the bacterial polysaccharide molecule is essential for the expression of type specificity and the capacity to induce antibacterial immunity, or whether the pattern unit, which in the case of the Type III Pneumococcus is cellobiuronic acid, will suffice, is the subject of the present investigation.

Two artificial azoprotein antigens have therefore been prepared, one containing the azobenzyl glycoside of the disaccharide cellobiose, the other the corresponding glycoside of cellobiuronic acid, the pattern unit of the Type III pneumococcus specific polysaccharide. The structural relationship of these two glycosides is represented by the following formulae:



p-aminobenzyl *β*-cellobioside



p-aminobenzyl β-cellobiuronide

These two substances differ only in the grouping occupying the 12th position which in the cellobiose is a primary alcohol grouping (CH_2OH) and in the cellobiuronide a carboxyl group (COOH). From the following account it will be seen that this slight difference in chemical constitution confers upon each antigen vastly different immunological properties.

EXPERIMENTAL

Methods

Immunization.—Two groups of rabbits weighing from 2 to 2.5 kilos were immunized respectively by the intravenous injection of 1 cc. of a sterile 0.5 per cent solution of the cellobiose and cellobiuronic acid antigens. The latter were prepared by combining the diazotized derivatives of the *p*-aminobenzyl glycosides of cellobiose and cellobiuronic acid to horse serum globulin as previously described (1 *c*). The animals received six daily doses of antigen and after a rest period of 7 days, the course of injections was repeated a second time. When necessary a third course of immunization was given. 7 days after the last injection the animals were bled from the ear and the sterile serum kept without preservative. In the tables the immunizing antigens will be referred to as cellobiose-globulin and cellobiuronic acid-globulin.

Technique of Immunity Reactions.—In the precipitin reactions, test antigens were prepared by combining the diazotized glycosides to the protein of chicken serum in order to avoid protein cross-reactions. In the agglutination reactions the antiserum to be tested was diluted with the appropriate quantity of sterile saline and an equal quantity of freshly prepared suspensions of heat-killed (70°) pneumococci of the specific type indicated in the protocols was added. All tubes were incubated at 37° for 2 hours and readings made after 24 hours in the ice chest.

The protection tests were done by the conventional technique; mice were injected intraperitoneally with 0.2 cc. of immune serum together with graded amounts of virulent cultures of pneumococci. The dilutions were so made that in all instances the total volume injected was 1 cc. Only those antisera having maximal precipitin titre for the homologous test antigen were used in mice, since antisera of lower titres failed to show appreciable protective action against virulent pneumococci. Following the course of intravenous injections the rabbits were tested for active immunity by the intradermal method of Goodner (4), using a culture of a rabbit virulent strain of Type III Pneumococcus, 0.001 cc. of which killed normal animals within 48 hours. The extent and character of the lesions, as well as the temperature of the animals, were recorded daily. All animals were observed for a period of 18 days before terminating the experiments.

RESULTS

Precipitins.—The immunization of the rabbits with the azoprotein antigens containing cellobiose and cellobiuronic acid was followed by means of the precipitin test. Two antisera obtained from each of two groups of rabbits which had received the cellobiuronic acid antigen were chosen for further investigation. Two cellobiose antisera likewise obtained from two groups of each of three rabbits were used in the immunological studies. All of these antisera yielded a marked precipitate with high dilutions of homologous test antigens.

Neufeld "Quellung" Reactions.—Using the standard technique for the Neufeld reaction it was found that a young actively growing culture of Type III Pneumococcus when mixed with cellobiuronic acid antiserum showed a typical and unmistakable swelling of the capsule indistinguishable from the Neufeld reaction produced by Type III antipneumococcus rabbit serum. The specificity of this reaction is the more striking since antisera to the cellobiose antigen failed to produce swelling of the capsule of Type III pneumococci. No swelling of the capsules of Types II and VIII pneumococci could be observed when the respective organisms were tested with cellobiose or cellobiuronic acid antisera. This point will be discussed further in the section dealing with the protective action of these sera.

Agglutinins.—The sera of rabbits injected with the cellobiose and cellobiuronic acid antigens were tested for agglutinins with heated suspensions of Types II, III, and VIII pneumococci. The results of typical experiments are given in Table I. From the results given in Table I it can be seen that the sera of rabbits immunized with the cellobiose antigen failed to agglutinate, in the range of dilutions used, any of the types of pneumococci tested. On the other hand, the cellobiuronic acid antisera in high dilutions agglutinated specifically Type III pneumococci but not the organisms of Types II and VIII. These experiments were carefully controlled in that the serum of the same animals obtained before immunization was similarly tested and in each instance found to be wholly devoid of specific antibodies for Type III pneumococci.

From the results of these experiments it can be concluded that the antisera of rabbits immunized with the artificial cellobiuronic acid antigen contain antibodies which cause swelling of the capsules and agglutination of Type III pneumococci, whereas the cellobiose antisera show neither of these properties. It is apparent, therefore, that the conversion of the primary alcohol group on the 12th carbon atom of cellobiose to the carboxyl group confers upon the cellobiuronic acid a new and important immuno-chemical function.

TABLE I

Agglutination of Types II, III, and VIII Pneumococci in Cellobiose and Cellobiuronic Acid Antisera

Antiserum prepared by immunization with	Pneumococcus Types	Final dilution of serum						
		1:5	1:10	1:20	1:40	1:80	1:160	1:320
Cellobiose-globulin	II	0	0	0	0	–	–	–
	III	±	0	0	0	–	–	–
	VIII	0	0	0	0	–	–	–
Cellobiuronic acid-globulin	II	0	0	0	0	–	–	–
	III	++	++	+++	+++	++	+	±
	VIII	±	0	0	0	0	0	0

Protective Antibodies: A. Cellobiuronic Acid Antiserum.—It has been found that the capsular polysaccharides of Types III and VIII pneumococci both contain cellobiuronic acid as an important constituent of the molecule (5). In order to determine whether sera of rabbits immunized with the artificial cellobiuronic acid antigen will confer passive immunity on mice against infection with these types of pneumococci, protection tests were performed by the technique described. For purposes of comparison tests against infection with Type I pneumococci were included in this experiment as well. Since the capsular polysaccharide of Type I Pneumococcus bears no structural similarity to that of Types III or VIII, one would not anticipate any protective action of the cellobiuronic acid antiserum against infection with organisms of Type I.

The results of the protection experiments given in Table II show that the serum of a rabbit immunized with the artificial cellobiuronic acid antigen is effective in protecting mice against infection with 10,000 and 1000 minimal lethal doses of Types III and VIII pneumococci respectively. As was anticipated, the anticellobiuronic acid serum failed to protect against infection with virulent Type I pneumococci.

TABLE II

*Protective Action of Anticellobiuronic Acid Rabbit Serum against Pneumococcus Infection in Mice**

Amount of culture	Pneumococcus							
	Type I		Type III			Type VIII		
cc.								
10^{-3}	—	—	D 48	D 48	S	—	—	—
10^{-4}	—	—	S	S	S	D 44	D 72	S
10^{-5}	D 40	D 40	S	S	S	S	S	S
10^{-6}	D 40	D 48	S	S	S	S	S	S
Virulence controls† (no serum)								
10^{-6}	D 40		D 32			D 28		
10^{-7}	D 48		D 48			D 28		
10^{-8}	D 48		D 48			S		

* The serum of a rabbit immunized with the first preparation of cellobiuronic acid antigen and showing the highest precipitin titre for the homologous test antigen was chosen for this experiment.

† The number of colonies developing in blood agar, seeded with the 10^{-7} and 10^{-8} dilutions were in all instances counted (Tables II to IV).

These experiments proved so striking that it was thought advisable to repeat them. Consequently an entirely new lot of the *p*-aminobenzyl glycoside of cellobiuronic acid was synthesized and the experiments repeated. The second preparation of antigen was administered as in the previous experiment to a group of three normal rabbits. After three courses of immunization one animal in this group failed to show cellobiuronic acid antibodies, a second gave a moderate antibody response, whereas the serum of the third animal showed the presence of precipitins in high titre and was used in the following protection tests.

This serum was tested in mice for the presence of protective antibodies against Pneumococcus Types II, III, and VIII and the results of these experiments are given in Table III. The experiments were controlled by including a group of mice which received virulent organisms together with the serum of the same rabbit before immunization with the cellobiuronic acid antigen was begun.

The results presented in Table III confirm the observations recorded in Table II and in addition show that the cellobiuronic acid antiserum affords protection against infection with Type II pneumococci as well as with Types III and VIII organisms. This result clearly demonstrates that the artificial antigen containing the azobenzyl glycoside of cellobiuronic acid stimulates in rabbits the formation of antibodies capable of conferring passive immunity on mice against infection with a number of different types of virulent pneumococci. The significance of this finding will be discussed later.

B. Cellobiose Antiserum.—It will be recalled that the chemical structure of the two saccharides, cellobiose and cellobiuronic acid, is identical save for the grouping occupying the 12th position in each. Antigens containing the azobenzyl glycosides of these two saccharides give rise in rabbits to antibodies which show some serological crossing, yet are quite specific as shown by inhibition tests (1 *c*). In the present investigation it has been found that the antiserum elicited by the cellobiuronic acid antigen agglutinates Type III pneumococci and causes a definite swelling of the capsule. The antiserum to the cellobiose antigen, on the other hand, exhibits neither of these phenomena.

In order, therefore, to ascertain whether the cellobiose antiserum will confer passive protection against pneumococcal infections, the most potent cellobiose antiserum was tested in mice with virulent cultures of Types II, III, and VIII pneumococci by the method described. In no instance was any protective action observed. The results of these experiments, which are given in Table IV, again demonstrate the wide variance in immunological function of the antibodies elicited by an antigen containing the disaccharide as opposed to the immune bodies evoked by the aldobionic acid antigen.

Active Immunity: A. Rabbits Injected with Cellobiose Antigen.—To ascertain whether the rabbits injected with the cellobiose antigen had acquired active immunity, six animals were infected, 12 days after the last injection of antigen, by the intradermal inoculation of 0.2 cc. of a blood broth culture of a rabbit virulent strain of Type III Pneumococcus. The virulence of the culture was such that 0.001 cc. injected intradermally killed normal control rabbits within 48 hours. The animals which had previously received the cellobiose antigen promptly developed massive edematous and necrotic lesions following infection and succumbed within 48 to 60 hours.

TABLE III

Protective Action of Anticellobiuronic Acid Rabbit Serum against Pneumococcus Infection in Mice[*]

Amount of culture	Pneumococcus					
	Type II		Type III		Type VIII	
cc.						
10^{-2}	D 72	S	D 24	D 24	—	—
10^{-3}	S	S	D 72	S	D 24	D 24
10^{-4}	S	S	S	S	D 40	D 72
10^{-5}	S	S	S	S	S	S
10^{-6}	—	—	—	—	S	S
Controls†						
10^{-6}	D 40		D 40		D 40	
10^{-7}	D 24		D 48		D 40	
10^{-8}	D 48		D 48		D 40	
Virulence controls (no serum)						
10^{-6}	D 40		D 40		D 40	
10^{-7}	D 24		D 48		D 40	
10^{-8}	D 40		D 48		S	

[*] The serum of a rabbit immunized with the second preparation of cellobiuronic acid antigen and showing the highest precipitin titre for the homologous test antigen was chosen for this experiment.

† Mice received 0.2 cc. of serum of rabbit before immunization with cellobiuronic acid antigen was begun.

B. Rabbits Injected with Cellobiuronic Acid Antigens.—Four of the rabbits which had received the cellobiuronic acid antigen were likewise tested for active immunity. The intradermal inoculation of virulent Type III organisms was made 12 days after the last injection of antigen. All four animals developed marked lesions and ran a febrile course. In each instance save one, however, the lesions were smaller and less edematous than in the normal controls or in the animals which had received the cellobiose antigen. Three of the infected rabbits recovered from the dermal infection. One rabbit died within 72 hours.

TABLE IV

*Protective Action of Anticellobiose Serum against Pneumococcus Infection in Mice**

Amount of culture	Pneumococcus					
	Type II		Type III		Type VIII	
cc.						
10^{-5}	D 28	D 45	D 45	D 45	D 45	D 45
10^{-6}	D 47	D 72	D 47	D 47	D 45	D 45
Virulence controls (no serum)						
10^{-6}	D 45		D 45		D 40	
10^{-7}	D 45		D 45		D 45	
10^{-8}	D 45		D 45		D 45	

* The serum of a rabbit immunized with cellobiose antigen and showing the highest precipitin titre for the homologous test antigen was chosen for this experiment.

The results of these experiments indicate clearly that rabbits immunized with the cellobiuronic acid antigen acquire definite resistance to intradermal infections with a virulent strain of Type III Pneumococcus. Animals injected with the cellobiose antigen, on the other hand, show no resistance whatsoever.

DISCUSSION

From the results of our immuno-chemical studies on uronic acid antigens, the concept has gradually evolved that it might be possible to confer on experimental animals immunity to pneumococcus infection with an artificial antigen containing a simple saccharide as the immuno-specific group instead of the more complex bacterial polysaccharide itself. Earlier studies showed that artificial antigens containing the azobenzyl glycosides of glucuronic and galacturonic acids, though reactive in antipneumococcal sera, failed to stimulate in various species of experimental animals immunity to pneumococcal infections (1 *b*). The reason for this failure may be attributed to the fact that the hexose uronic acids do not approximate closely enough in structure the aldobionic acids which constitute the fundamental building stones of certain of the type specific polysaccharides of bacterial origin. The structural unit of the Type III pneumococcus polysaccharide is cellobiuronic acid (2 *a*). This aldobionic acid is unusually suited for testing the hypothesis set forth above, since much of the basic research for such a study already has been accomplished, and the acid itself is readily procured from the acid hydrolysis products of the bacterial polysaccharide. From the results of the present investigation it has been proven beyond question that the aldobionic acid, functioning as the immuno-specific group of an artificial antigen, evokes in rabbits antibodies which have many properties in common with those elicited by an antigen containing the more complex capsular polysaccharide.

In a communication presented some years ago from this laboratory (3), it was shown that an artificial antigen containing the azobenzyl ether of the Type III capsular polysaccharide evoked in rabbits antibodies which specifically agglutinated Type III pneumococci, precipitated the homologous specific polysaccharide, and protected mice against infection with Type III organisms. Not only does the cellobiuronic acid antiserum precipitate the Type III capsular polysaccharide, when combined with egg albumin (1 *c*), and agglutinate Type III organisms, but the sera of animals immunized with the cellobiuronic acid antigen likewise confer passive protection on mice against infection with virulent Type III pneumococci.

It should not be inferred, however, that the antibodies evoked by the polysaccharide antigen or by heat-killed Type III pneumococci are identical with those elicited by the cellobiuronic acid antigen. The results of the specific inhibition tests presented in the previous communication (1 *c*) clearly demonstrate that the polysaccharide and cellobiuronic acid antibodies are similar but not identical since they fail to show a complete reciprocal relationship. Furthermore the results of the experimental studies presented in this communication have brought forth a new and important principle. Whereas the antigen containing the complex bacterial Type III pneumococcus carbohydrate gives rise to antibodies which are type specific, those elicited by the antigen containing the pattern unit, or aldobionic acid show a broader specificity for they confer passive protection on mice not only against infection with Type III pneumococci but against Types II and VIII organisms as well.

Although cellobiuronic acid antiserum causes agglutination and *Quellung* only with the Type III Pneumococcus it must be borne in mind that protection tests are far more subtle than are these gross qualitative phenomena and that protection can be demonstrated with amounts of antibody which cannot be detected with other techniques. Furthermore, it has been proven that cellobiuronic acid is a constituent of the Type VIII pneumococcus polysaccharide. For these reasons, therefore, it is not out of the question that the protection which cellobiuronic acid antiserum affords mice against infection with Type VIII pneumococci can be attributed to the identity in structure of a portion of the polysaccharide molecule. The striking results obtained with Type II Pneumococcus cannot be explained until a more comprehensive understanding of the uronic acid constituent of the capsular polysaccharide of this microorganism is had. The results of the foregoing experiments indicate the importance of ascertaining the exact constitution of the specific polysaccharides of encapsulated pathogens, for it is only through such knowledge that the enigma of their specificities will be fully explained.

SUMMARY

1. An artificial antigen containing the azobenzyl glycoside of cellobiuronic acid gives rise in rabbits to antibodies which: (*a*) give the Neufeld reaction and agglutinate Type III pneumococci, (*b*) confer passive protection on mice against infection with Types II, III, and VIII pneumococci.

2. Rabbits immunized with the artificial cellobiuronic acid antigen acquire active resistance to infection with virulent Type III pneumococci.

3. The antibodies evoked by an antigen containing the azobenzyl glycoside of cellobiose exhibit none of these phenomena.

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2. (*a*) Hotchkiss, R. D., and Goebel, W. F., *J. Biol. Chem.*, 1937, **121**, 195. (*b*) Heidelberger, M., and Goebel, W. F., *J. Biol. Chem.*, 1927, **74**, 613.
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SACCHAROLACTONE METHYL ESTER

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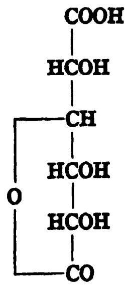
Crystalline saccharolactone (I) recently has been proved by Schmidt and Günthert[1] to be the 3,6-monolactone of saccharic acid. On mild treatment with diazomethane saccharolactone was found to yield a crystalline methyl ester (II), m. p. 113–114°. The structure of the ester was established by applying the procedure of Jackson and Hudson[1a] as used by Schmidt and Günthert: namely, the isolation of oxalic and *d*-tartaric acids following periodic acid cleavage (between C_4—C_5) and subsequent oxidation with bromine in the presence of calcium carbonate.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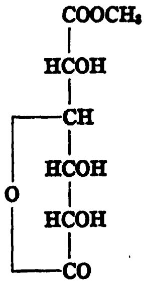
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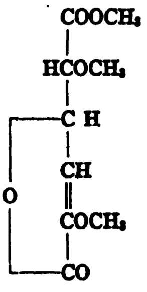
III

Saccharolactone methyl ester (II) may be saponified to potassium acid saccharate, or converted to saccharic acid diamide. It gives a crystalline benzylidene compound whose structure has not been determined. The ester also reacts further with ethereal diazomethane

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(1) O. Th. Schmidt and P. Günthert, *Ber.*, 71, 493 (1938).

(1a) E. L. Jackson and C. S. Hudson, THIS JOURNAL, 59, 994 (1937).

to give the unsaturated lactone (III), m. p. 87°, which was previously described by Schmidt, Zeiser and Dippold.[2]

EXPERIMENTAL

All melting points reported in this paper were observed in a modified Fischer–Johns apparatus mounted on the stage of a polarizing microscope. Specific rotations were determined with the D-line of sodium light and at 26° unless otherwise specified.

Saccharolactone Methyl Ester.—Five grams of saccharolactone,[3,4] m. p. 132–134°,[5] was dissolved at room temperature in 350 cc. of dry methanol, and the solution immediately cooled in an ice-bath. To this was added a cold ethereal solution of diazomethane until a faint yellow coloration persisted for a few minutes after thorough mixing. The diazomethane obtained from 25–30 g. of nitrosomethyl urea was required. The excess reagent was removed by addition of a trace of glacial acetic acid, and the solution rapidly concentrated to dryness under diminished pressure at 20°. The resulting sirup was dissolved in 10 cc. of absolute alcohol which upon standing in the ice-chest deposited 3.23 g. of crystalline saccharolactone methyl ester. After recrystallization from absolute alcohol, the product melted at 113–114°; sp. rot. +29.0° (c, 0.8, H_2O).

Anal. Calcd. for $C_7H_{10}O_7$: C, 40.78; H, 4.85; OCH_3, 15.1; eq. wt., 103. Found: C, 40.91; H, 4.30; OCH_3, 15.5; eq. wt., 105.8.

The product reduces Fehling's solution, in contrast with the action of saccharolactone. Quantitatively,[6] the reduction amounts to less than 1% of that of glucose. On standing for several months in a desiccator at room temperature the substance decomposes to form a yellow gum. It was noticed that a faint yellow coloration appeared whenever the crystals were dried in high vacuum.

Saponification of Saccharolactone Methyl Ester to Yield Potassium Acid Saccharate.—Saccharolactone methyl ester (200 mg.) was boiled for a few minutes with 2.2 cc. of N potassium hydroxide. Several drops of glacial acetic acid and a trace of Norite were added and the solution filtered. On cooling 162 mg. of potassium acid saccharate separated; sp. rot. +6.25° → + 19.2° (twenty days in water containing 1 eq. HCl).

Anal. Calcd. for $C_6H_9O_8K$: K, 15.77. Found: K, 15.97.

Preparation of Saccharic Acid Diamide from Saccharolactone.—A solution of 260 mg. of saccharolactone in 2 cc. of methanol-ammonia (saturated at 0°) quickly

[2] O. Th. Schmidt, H. Zeiser and H. Dippold, *Ber.*, **70**, 2402 (1937).

[3] H. Kiliani, *ibid.*, **58**, 2344 (1925).

[4] K. Rehorst and H. Scholz, *ibid.*, **69**, 520 (1936).

[5] A lower melting sample of saccharolactone failed to yield the crystalline ester until purified by recrystallization from ether.

[6] Hanes modification of the Hagedorn–Jensen method, C. S. Hanes, *Biochem. J.*, **23**, 99 (1929).

deposited a gum which crystallized on standing at 0°. After recrystallization from 50% alcohol the saccharic acid diamide[7] melted at 176–178°; yield 140 mg.

Benzylidene Derivative of Saccharolactone Methyl Ester.—Saccharolactone methyl ester, 1.2 g., was shaken with 1 g. of anhydrous zinc chloride and 6 cc. of benzaldehyde. The mixture soon solidified, at which time 10 cc. of benzene was introduced and the shaking continued for five hours. Petroleum ether was stirred into the mixture which was then filtered and washed with petroleum ether. The precipitate was then washed on the filter with water until free from chlorine ion. After recrystallization from methanol, m. p. 237–238°, yield 0.80 g.; sp. rot. +147° (*c*, 1.0 in pyridine).

Anal. Calcd. for $C_{14}H_{15}O_7$: C, 57.06; H, 5.09; OCH_3, 10.50; eq. wt., 147. Found: C, 57.00; H, 4.84; OCH_3, 11.13; eq. wt., 149.

The product is insoluble in water, ether, petroleum ether and benzene, difficultly soluble in hot acetone, absolute alcohol, ethyl acetate and methanol. It dissolves slowly in aqueous sodium hydroxide with liberation of benzaldehyde (odor).

Periodic Acid Cleavage of Saccharolactone Methyl Ester Followed by Oxidation to Yield Oxalic and d-Tartaric Acids.—To 618 mg. (3 mol.) of saccharolactone methyl ester in 3 cc. of water was added 10 cc. of 0.3 *M* periodic acid solution. After one hour at room temperature 7.5 cc. of 2 *N* hydriodic acid was introduced and the mixture shaken, filtered and extracted with chloroform to remove iodine. The aqueous solution was then neutralized with a slight excess of solid calcium carbonate and, under good stirring, 3 g. more of calcium carbonate was added with 1.5 cc. of bromine. The solution no longer reduced Fehling's solution. The precipitated calcium salts were removed by centrifugation and dissolved in 5 cc. of hot water by the dropwise addition of concd. hydrochloric acid. The solution was then neutralized to methyl orange by addition of sodium acetate and the precipitated calcium oxalate was collected and washed by centrifugation; yield 142 mg.

Anal. 66.7 mg. required 10.29 cc. 0.1 *N* permanganate. Calcd., 10.42 cc. 0.1 *N* permanganate.

The supernatant solution from the calcium oxalate precipitate was neutralized to phenolphthalein with 2 *N* sodium hydroxide. The precipitated calcium tartrate was collected and washed by centrifugation; yield 263 mg.

Anal. Calcd. for $C_4H_4O_6Ca \cdot 4H_2O$: Ca, 15.37. Found: Ca, 15.58.

A suspension of the calcium tartrate was shaken with the calculated amount of oxalic acid, filtered and on evaporation to dryness yielded crystalline *d*-tartaric acid, m. p. 160°; sp. rot. +14.8° (H_2O).

Unsaturated Lactone Ester (III) Obtained by the Action of Diazomethane.—Saccharolactone methyl ester, 315 mg., was treated with 20 cc. of 0.7 *M* diazomethane in ether, and allowed to stand in the ice-chest for one week. The supernatant solution was decanted from the crystals which separated; yield 142 mg.;

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(7) M. Bergmann, *Ber.*, 54, 2651 (1921).

m. p. 87–88°; sp. rot. (21°) + 79.5° (*c*, 1.25 in methanol). Schmidt, Zeiser and Dippold[8] report for compound III, m. p. 87°, sp. rot. (20°) + 83.1° (methanol).

SUMMARY

The methyl ester of saccharolactone <3,6> has been prepared and its structure proved by periodic acid cleavage followed by oxidation of the product to yield oxalic and *d*-tartaric acids.

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ANTIGENIC PROPERTIES OF THE TYPE-SPECIFIC SUBSTANCE DERIVED FROM GROUP A HEMOLYTIC STREPTOCOCCI

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Although the constituent of the hemolytic streptococcus cell, which determines specificity in each of the various types included within group A, has been considered a protein, attempts to demonstrate its antigenicity after separation from the cell have not been entirely successful. This paper gives a more detailed study of this subject. It seems probable that in some instances the chemical procedures used to isolate the type-specific substances tended to degrade the proteins, and thus render them less antigenic. The early experiments (1) with the type-specific protein M showed that although the sera produced by the injection of whole streptococci gave specific precipitin reactions with this partially purified extract, the injection of the protein solution itself failed to stimulate type-specific antibody production in rabbits or guinea pigs, as shown by the following negative tests: precipitin reaction, agglutination, passive protection in mice, passive and active anaphylaxis in guinea pigs.

By extracting ground streptococci with increasingly alkaline solutions, Heidelberger and Kendall (2) isolated a fraction which stimulated type-specific precipitin formation when injected into rabbits; but these sera were not tested for their ability to protect mice passively.

Mudd and his collaborators (3) have reported the isolation of an antigenic fraction from group A hemolytic streptococci which they have named labile antigen.[1] They postulated that the labile antigen is a complex molecule containing the type-specific protein M in addition to several other serologically active constituents. Besides absorbing type-specific agglutinins, opsonins, and protective antibodies from antibacterial serum, the labile antigen, when injected into rabbits, induced antibodies which gave partly type-specific precipitin reactions, but these antisera were not tested for protective action in animals.

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[1] On the basis of recent unpublished findings of Mudd, Lackman, Pettit, and Morgan, Dr. Mudd has informed us that these authors now prefer to substitute the term "nucleoprotein agglutinogen" for "labile antigen."

In 1937, Stamp and Hendry (4) isolated a fraction from a group A type 3 hemolytic streptococcus (strain Richards), which produced active immunity when injected into mice. 47 per cent of the immunized animals were protected against 100 minimal lethal doses of the homologous organism. The specificity of this immune response, however, was not tested.

EXPERIMENTAL

As a starting point for obtaining the type-specific substance in antigenic form, it was thought promising to use bacteria ground in the cold, as suggested by Mudd and his collaborators. The first untreated saline extracts of such material, injected into rabbits, induced the formation of slight amounts of antibody, as demonstrated by precipitin and agglutinin tests and the passive protection of mice, but the antibody titers of these sera were very low.

Because of the large quantities of extract required to immunize rabbits, it was decided to immunize mice and to test their active immunity following the method of Stamp and Hendry, using the per cent of survival of actively immunized mice as the index of antigenicity of various preparations. In numerous experiments this technique proved to be a rough but convenient way of testing various fractions.

Methods

Selection of Strains.—Since the antigenicity of the extracts was to be tested by actively immunizing mice, it was essential to use only highly mouse-virulent strains which would be suitable for testing the degree of immunity in mice. It seemed probable, furthermore, that virulent cultures would yield larger amounts of antigenic material than avirulent ones. One strain each of three different types within group A was used for preparing antigenic extracts and one additional strain of each of these types was employed for testing the immunity.

Description of Strains.[2]—

Type 1:[3] 1. *Strain S118* was isolated in Texas in 1918 from the pleural fluid of a patient with bronchopneumonia following measles (6).

2. *Strain T1* is Griffith's type 1 strain, S. F. 130/2 (7).

Type 3: 1. *Strain D58* is the strain Richards, isolated by Colebrook from puerperal septicemia (4). It was obtained in a virulent state following mouse passage.

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[2] Strains T1 and T14 were kindly sent by Dr. F. Griffith, strain D58 by Dr. T. C. Stamp, and strain C203 by Dr. M. B. Kirkbride.

[3] Types are designated according to Griffith's classification (5).

2. *Strain C203*[4] was isolated by Dochez about 1921 from a patient with scarlet fever, and obtained in virulent form in 1927.

Type 14: 1. *Strain S23* was isolated in Texas in 1918 from the throat of a patient with lobar pneumonia (6).

2. *Strain T14* is strain Barker, a representative of type 14.

Virulence.—At the beginning of this experiment, three of the six strains, namely S23, C203, and D58, were virulent enough to kill mice in doses of 10^{-6} cc. to 10^{-8} cc. of 6 to 12 hour cultures. Strain T14 was moderately virulent and required only six to eight mouse passages to reach the same degree of virulence. The other two strains, S118 and T1, were so degraded that they produced typically glossy colonies and failed to yield demonstrable type-specific substance in extracts of ordinary concentration. They were so avirulent that 0.1 cc. to 0.5 cc. of a young culture was required to kill mice. By repeated mouse passage (25 passages for S118 and 60 passages for T1) it was possible to render these cultures virulent.

The chief method used in the preparation of antigenic extracts was as follows:—

Medium.—The bacteria used for preparing antigenic extracts were grown in the type of broth developed by Todd and Hewitt (8), modified chiefly by the substitution of beef heart for horse meat. The fact that the broth is sterilized by filtering through Chamberland B filters, rather than by heating, increases its value as a medium but also adds an element of danger in its use, since contaminants sometimes grow in uninoculated flasks which have been incubated for 3 or 4 days. By seeding the broth immediately after filtration with a very large inoculum, and then limiting the incubation to 4 hours, pure cultures were obtained in all except one instance.

During the early stages of growth in this broth, large capsule-like areas could be seen surrounding the organisms in moist India ink preparations. These capsules increased markedly in size and were maximal in about 4 hours, following which they rapidly became smaller. The bacteria at this early stage were very difficult to throw down in the centrifuge, but following heating at 56°, or after more prolonged incubation, the capsules disappeared and the bacteria were then easily packed on centrifugation, an observation also made by Seastone (9) and more recently by Loewenthal (10).

These capsules resembled those described by Seastone and later by Ward and Lyons (11), in young cultures of hemolytic streptococci grown in whole blood, in that they were present only in young cultures and were not correlated with mouse virulence, but they differed in that we could not stain them by ordinary methods. The difficult question of the significance of capsules for the hemolytic streptococcus has been reviewed by a number of authors (9, 10, 12), but in these experiments the presence of a capsule was taken to mean that the cultures were in a period of optimal physiological activity.

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[4] While Dr. Griffith has placed this strain in type 1 by means of slide agglutination, work carried out in this laboratory indicates that, on the basis of protection and precipitin tests, it falls in type 3. Further studies are being made here on the interrelationships of strain C203 and types 1 and 3.

Collection of Bacteria.—The broth was filtered while still hot through Chamberland filters and was then cooled to 37°C. 64 liters were inoculated with 25 cc. per liter of an actively growing 4 hour culture. After 4 hours' growth, samples were removed from each flask and plated on blood agar to test the purity of the culture. The culture was then stored at 0°C. overnight, and kept chilled while running through a Sharples centrifuge on the following day. The caked bacterial sediment was suspended in about 200 cc. of cold saline by grinding it in a large, previously chilled mortar and pressing it through sterile gauze, stretched over a wire strainer, into another cold container in order to break up clumps and facilitate uniform heating during the process of heat-killing. The suspension was then transferred to a glass stoppered pyrex bottle, and the bacteria killed quickly by the following procedure: A thermometer was inserted into the bottle so that the temperature could be accurately adjusted. The temperature was raised within 1 minute to 56°C. by the addition, while shaking, of about 400 cc. of boiling saline. The stopper was then covered tightly with a rubber cap; and the bottle was completely immersed for 15 minutes in a 56°C. water bath. Sterility tests showed that all the bacteria in this heavy suspension were killed by the end of this time. The suspension was rapidly cooled by placing the bottle under running water and then centrifuged in 50 cc. wide mouthed tubes; the supernatant fluid was discarded. The tubes, containing not more than 3 gm. dry weight of bacterial sediment, were placed in a CO_2-ice-acetone mixture. While freezing, the bacteria were easily distributed over the sides of the tube with a spatula. The completely frozen organisms were dried by means of the Flosdorf-Mudd lyophile apparatus (13).

Extraction.—Preparatory to extraction, the bacteria were ground in a ball mill consisting of a 1 liter spherical heavy glass flask and 500 one-quarter inch stainless steel balls (14). At first the grinding was done at −73°C. as advocated by Mudd (15). Later, however, it was found that satisfactory results could be obtained by grinding 1 gm. of dried organisms in each flask for one-half hour at room temperature. This procedure rendered approximately 75 per cent of the cocci Gram-negative without causing much change in their morphology. The organisms from twelve flasks were collected in 500 cc. N/10 HCl and extracted at 37°C. for 24 hours. After centrifugation the supernatant extract was removed; 250 cc. N/10 HCl were added to the organisms which were then extracted for a second 24 hours. This procedure was repeated on a 3rd day. The yield of active material from the second and third extractions tended to be larger than that from the first; but since further extractions resulted in smaller yields, the bacterial residue was discarded.

Purification of the Extract.—On cautious neutralization of each of these acid extracts with N/1 NaOH a precipitate began to form as pH 4.0 was approached and became maximal at about pH 4.5. In the case of the first acid extract it was necessary to bring the pH to 5.0 before flocculation occurred. After standing overnight in the ice box the precipitates were thrown down in a centrifuge and the supernatant fluids discarded. The combined precipitates from various extractions were taken up in M/15 phosphate buffer solution at pH 7.2 but most of the precipitate was insoluble and was discarded. N/1 HCl was slowly added to the supernatant fluid. As pH 5.5 was approached a precipitate began to form which was maximal at pH 4.5. This was allowed to stand overnight in the ice box and the precipitate separated the following morning. The supernatant fluid was discarded, and now nearly all the precipitate was dissolved in 50 cc. of M/15 phosphate buffer at pH 7.2. After removal of the small amount of insoluble material the solution was filtered through a Berkefeld N filter.

The filtrate was distributed in amounts suitable for one day's injections and then frozen and dried to prevent deterioration. In order to estimate the dosage, the total nitrogen and that precipitated by trichloroacetic acid were determined by means of the micro Kjeldahl method. The nitrogen precipitated by trichloroacetic acid was always between 50 and 60 per cent of the total nitrogen. Since it was felt that the antigenic activity probably resided in the protein fraction, the dosage in all experiments was calculated as 6.25 times the nitrogen content of the material precipitated by trichloroacetic acid; and the total yield calculated in the same way was about 0.6 per cent of the dry weight of the bacteria.

Serological Reactions Obtained with the Extract.—Different preparations of antigen reacted type specifically to about the same dilution, usually 1:200,000 in precipitin tests with homologous type-specific sera. The dilution was calculated in the same way as the dosage employed in immunization. No group-specific polysaccharide C could be detected in these extracts when tested with sera potent in anti-C precipitins nor could this carbohydrate be split off by heating the solution at 100°C. with N/20 HCl.

The method of extraction and partial purification outlined was adopted as the procedure of choice for the chief experiments of this investigation, but several less successful methods of preparation were tried in numerous other experiments.

Active Immunization of Mice

The mice used in these experiments were the Rockefeller strain. When possible, mice weighing 24 to 26 gm. were used for active immunization, since they seemed to give better results than smaller ones. The dried antigenic extracts were dissolved in physiological sodium chloride solution just before injection, which was made intraperitoneally on 3 successive days with a rest period of 4 days between courses. Usually three or four courses were given. The immunized and untreated control mice were inoculated with the test culture 1 week after the last injection of the antigen. Experiments were terminated after 2 weeks' observation.

The mice in the first experiment (Table I) were immunized for 4 weeks with an

TABLE I

Active Protection Test in Mice

Titer of Homologous Immunity

Mouse No.	Mice immunized with extract of type 1 strain, S118						Untreated controls	
	Inoculated with the following amounts of S118							
	10^{-2} cc.	10^{-3} cc.	10^{-4} cc.	10^{-5} cc.	10^{-6} cc.	10^{-7} cc.	10^{-7} cc.	10^{-8} cc.
1	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day
2	D 1 "	D 1 "	D 2 days	D 1 "	D 3 days	D 3 days	D 1 "	D 1 "
3	D 1 "	D 1 "	D 2 "	D 1 "	D 3 "	D 5 "	D 1 "	D 1 "
4	D 1 "	D 1 "	D 4 "	D 2 days	D 5 "	S	D 1 "	D 1 "
5	D 1 "	D 1 "	D 6 "	D 2 "	D 6 "	S	D 1 "	D 1 "
6	D 1 "	D 1 "	D 7 "	D 2 "	S	S	D 1 "	D 1 "
7	D 1 "	D 1 "	D 10 "	D 3 "	S	S	D 1 "	D 1 "
8	D 1 "	D 1 "	S	D 3 "	S	S	D 1 "	D 1 "
9	D 1 "	D 1 "	S	D 3 "	S	S	D 1 "	D 1 "
10	D 1 "	D 1 "	S	D 3 "	S	S	D 1 "	D 1 "
11	D 1 "	D 1 "	S	D 3 "	S	S	D 1 "	D 1 "
12	D 1 "	D 3 days	S	S	S	S	D 1 "	D 1 "
13	D 1 "	D 4 "	S	S	S	S	D 2 days	D 1 "
14	D 1 "	S	S	S	S	S	D 2 "	D 2 days
15	D 1 "	S	S	S	S	S	D 3 "	D 3 "
16	D 1 "	S	S	S	S	S	D 3 "	D 3 "
17	D 1 "	S	S	S	S	S	S	D 3 "
18	D 1 "	S	S	S	S	S	S	D 3 "
19	D 1 "	S	S	S	S	S	S	D 5 "
20	D 1 "	S	S	S	S	S	S	S

The mice were immunized for 4 weeks: 3 injections of 0.01 mg. each were given the 1st week, 3 of 0.02 mg. each the 2nd, 3 of 0.04 mg. each the 3rd and 4th.

The protection tests were performed as follows: A fresh 16 hour broth culture was serially diluted with broth so that the amount inoculated was contained in 0.5 cc. The inoculations were intraperitoneal. The untreated controls corresponded in age and weight with the immunized animals. The number of streptococci injected was estimated in colony counts from poured blood agar plates containing 10^{-6} cc., 10^{-7} cc., and 10^{-8} cc., respectively, of the culture used for inoculating the mice. The number of colonies in 10^{-8} cc. varied from two to six in different experiments.

In all experiments S indicates animals which survived at least 2 weeks, and D indicates death on the day stated.

extract [illegible]rain S118 in the manner indicated in Table I. A total dosage of 0.33 mg. was given to each animal. 1 week after the last injection, they were divided into six groups of 20 mice each, and each group was injected with a different dose of an overnight culture of strain S118. None of the mice receiving 1,000,000 M.L.D. (10^{-2} cc.) survived, but in all the groups receiving smaller doses enough animals survived to show definite protection against the homologous organism.

The second experiment (Table II) was designed to test whether the active immunity induced by these antigens was type-specific. Three sets of 84 mice were immunized for four courses with extracts of streptococci of three different types (types 1, 3, and 14). The mice received the same amounts of antigenic extract as those in the first experiment. 1 week following the last dose of antigen each group was subdivided into six subgroups of 14 mice each. Six strains were used as test inocula, two each of types 1, 3, and 14. Each subgroup received 10^{-6} cc. of culture diluted in broth. This small dose, containing 10 to 100 M. L. D., which killed all the control animals regularly, was selected in order to detect even slight evidence of cross protection.

In each case, good protection was demonstrated against 10 to 100 lethal doses of streptococci of the homologous type, as there were 93 per cent survivors in type 1, 93 to 100 per cent in type 3, and 43 to 50 per cent in type 14. On the other hand, comparably immunized mice, when inoculated with heterologous strains, showed only slight or no immunity. Some protection against the type 1 cultures was afforded to mice immunized with heterologous extracts, but little if any cross immunity was found where types 3 and 14 were used as test inocula. In every case type-specific immunity was clearly greater than the immunity against heterologous types. It is probable that if a larger dose of culture had been used, the immunity induced by immunization with extracts would have appeared strictly type-specific.

Type-Specific Immunization of Rabbits with Extract

After completing the experiments on active immunity in mice, an attempt was made to immunize rabbits with the same antigenic extract. The immunity was tested by protection of mice by the sera of these animals. The rabbits were treated as shown in Table III. During immunization the response to the antigen was determined by precipitin tests with the homologous M extract.

These tests showed that the sera of the two rabbits immunized with S118 extract (rabbits R47-08 and R47-09) contained no precipitins against the homologous extract at the end of the third course, but both had precipitins and protective antibodies at the end of the fifth course. The rabbit given seven courses showed no increase in antibody titer over that reached at the end of the fifth series. The sera of the two rabbits (R47-22 and R47-23) similarly immunized with an

TABLE II

Active Protection Test in Mice

Type Specificity of Immunity

Immunized with extract of strain	Mouse No.	Immunized mice					
		Inoculated with 10^{-6} cc. of					
		Type 1 strains		Type 3 strains		Type 14 strains	
		S118	T1	D58	C203	S23	T14
		(Homologous)					
S118 (type 1)	1	D 7 days	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day
	2	S	S	D 1 "	D 1 "	D 1 "	D 1 "
	3	S	S	D 1 "	D 1 "	D 1 "	D 1 "
	4	S	S	D 1 "	D 1 "	D 1 "	D 1 "
	5	S	S	D 1 "	D 1 "	D 1 "	D 2 days
	6	S	S	D 1 "	D 1 "	D 2 days	D 2 "
	7	S	S	D 1 "	D 1 "	D 2 "	D 2 "
	8	S	S	D 2 days	D 1 "	D 3 "	D 2 "
	9	S	S	D 2 "	D 1 "	D 3 "	D 3 "
	10	S	S	D 2 "	D 2 days	D 3 "	D 3 "
	11	S	S	D 2 "	D 2 "	D 5 "	D 3 "
	12	S	S	D 2 "	D 2 "	D 5 "	D 3 "
	13	S	S	D 3 "	D 2 "	D 9 "	D 3 "
	14	S	S	D 3 "	D 2 "	S	D 5 "
				(Homologous)			
D58 (type 3)	1	D 1 day	D 1 day	S	D 3 days	D 1 day	D 1 day
	2	D 1 "	D 1 "	S	S	D 1 "	D 2 days
	3	D 1 "	D 1 "	S	S	D 1 "	D 2 "
	4	D 2 days	D 2 days	S	S	D 1 "	D 2 "
	5	D 2 "	D 2 "	S	S	D 2 days	D 2 "
	6	D 2 "	D 2 "	S	S	D 2 "	D 2 "
	7	D 2 "	D 2 "	S	S	D 2 "	D 2 "
	8	D 2 "	D 2 "	S	S	D 2 "	D 2 "
	9	D 3 "	S	S	S	D 3 "	D 2 "
	10	D 3 "	S	S	S	D 3 "	D 2 "
	11	S	S	S	S	D 3 "	D 3 "
	12	S	S	S	S	D 7 "	D 3 "
	13	S	S	S	S	D 7 "	D 3 "
	14	S	S	S	S	S	D 8 "
						(Homologous)	
S23 (type 14)	1	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day
	2	D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	D 2 days
	3	D 1 "	D 1 "	D 1 "	D 1 "	D 2 days	D 2 "
	4	D 1 "	D 1 "	D 1 "	D 1 "	D 5 "	D 2 "
	5	D 1 "	D 1 "	D 1 "	D 1 "	D 5 "	D 3 "
	6	D 2 days	D 2 days	D 1 "	D 1 "	D 6 "	D 3 "
	7	D 2 "	D 2 "	D 1 "	D 1 "	D 7 "	D 3 "
	8	D 2 "	D 2 "	D 1 "	D 1 "	S	D 9 "
	9	D 2 "	D 2 "	D 1 "	D 1 "	S	S
	10	D 2 "	D 2 "	D 1 "	D 2 days	S	S
	11	D 2 "	D 3 "	D 1 "	D 2 "	S	S
	12	S	D 3 "	D 1 "	D 2 "	S	S
	13	S	S	D 2 days	D 2 "	S	S
	14	S	S	D 2 "	D 2 "	S	S

TABLE II—*Concluded*

Untreated virulence control mice

Mouse No.	Dose	Inoculated with					
		Type 1 strains		Type 3 strains		Type 14 strains	
		S118	T1	D58	C203	S23	T14
	cc.						
1	10^{-6}	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day
2		D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	D 1 "
3		D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	D 1 "
4		D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	D 1 "
5		D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	D 1 "
6		D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	D 1 "
7		D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	D 2 days
8		D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	D 2 "
9		D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	D 2 "
10		D 1 "	D 1 "	D 1 "	D 2 days	D 2 days	D 2 "
11		D 1 "	D 1 "	D 1 "	D 2 "	D 2 "	D 2 "
12		D 1 "	D 1 "	D 1 "	D 2 "	D 2 "	D 2 "
13		D 1 "	D 1 "	D 1 "	D 2 "	D 2 "	D 2 "
14		D 5 days	D 1 "	D 2 days	D 15 "	D 2 "	D 7 "
1	10^{-7}	D 1 day	D 1 "	D 1 day	D 1 day	D 1 day	D 2 "
2		D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	D 2 "
3		D 1 "	D 1 "	D 1 "	D 1 "	D 2 days	D 2 "
4		D 1 "	D 1 "	D 1 "	D 2 days	D 2 "	D 2 "
5		S	D 1 "	D 3 days	D 9 "	S	D 2 "
1	10^{-8}	D 1 day	D 2 days	D 1 day	D 2 "	D 2 days	D 1 day
2		S	D 2 "	D 1 "	D 2 "	S	D 2 days
3		S	D 2 "	D 2 days	D 2 "	S	D 2 "
4		S	S	D 2 "	D 2 "	S	D 2 "
5		S	S	S	S	S	S

extract of strain D58, contained demonstrable type-specific precipitins and protective antibodies after 3 weeks' immunization; and the titer was increased following 2 weeks' further immunization.

The capacity of these sera to protect mice was tested with six different strains of hemolytic streptococci, representing three types (Table IV). The S118 serum was a pool of bleedings from rabbit R47-08 after the fifth and seventh courses of injections of antigen. The D58 serum was taken from rabbit R47-23 after the fifth course. Although the cultures used in the passive protection experiments were somewhat less virulent than usual, as shown by the survival of some control mice, nevertheless, there was distinct protection against 1,000 to 100,000 M.L.D. of the homologous type streptococci with some irregular deaths. The few survivals among animals tested with heterologous strains were very irregular, and the results in general show strict type specificity in these passive protection tests.

TABLE III

Protocol of Immunization of Rabbits with Extracts

Course of injection	Rabbits R47-08 and R47-09* with S118 extract	Rabbits R47-22 and R47-23 with D58 extract
1st	2.5 mg. daily for 5 days	2.5 mg. daily for 5 days
2nd	4.3 " " " " "	2.5 " " " " "
3rd	5.0 " " " " "	5.0 " " " " "
	Test bleeding	Test bleeding
4th	10.0 mg. daily for 5 days	5.0 mg. daily for 5 days
5th	10.0 " " " " "	10.0 " " " " "
	50 cc. bleeding	Final bleeding
6th	10.0 mg. daily for 5 days	
7th	20.0 " " " " "	
	Final bleeding	

Immunizing material was dissolved in saline, 1 to 2 cc. and given intravenously. There were 2 days of rest between each course except where a bleeding was taken, in which case the interval was a week.

* The final bleeding from rabbit R47-09 was taken after the sixth course of injections.

Precipitin Reactions with Anti-Extract Sera

In Table V are shown the precipitin reactions with samples of the same sera used in the passive protection tests recorded in Table IV. The M substances used as reagents were extracted with N/20 HCl in a boiling water bath as previously described (16). The immediate reactions were strikingly type-specific, but on standing overnight in the ice box the somewhat confusing cross reactions appeared. The latter are recorded in the table. The type 1 serum was not very potent but reacted most strongly with extracts of the homologous type strains, S118 and T1. It also gave weak reactions with all three of the type 3 extracts used but none with the type 14 extracts. The much more potent type 3 serum gave good immediate precipitates with extracts of all the homologous type strains (T3, D58, C203), and weaker reactions with the type 1 extracts. With type 14 extracts, the type reactivity of which had been previously established by testing with antibacterial sera, this serum (R47-23) gave only traces of precipitin reaction. Neither serum contained group-specific antibody, as indicated by their failure to precipitate with a solution of group-specific C polysaccharide which, in the dilutions used, regularly precipitated sera known to contain the group-specific anti-C precipitin.

It is highly probable that the cross reactions in the precipitin tests are due to the presence of non-type-specific antibodies in the rabbit serum and of non-type-specific precipitinogens in the M extracts used.

Absorption of Antibacterial Serum with Extracts

In order to test the evidence identifying this antigen with the substance in intact streptococci which stimulates the production of protective antibodies in rabbits, the antigen was used to absorb protective antibody from a serum made by immunizing a rabbit with whole streptococci. Since Mudd and his collaborators (3) state that the type-specific protective antibody cannot be absorbed by the M fraction, extracted with N/20 HCl in a boiling water bath, another sample of the serum was absorbed with the M substance so prepared.

A rabbit was immunized chiefly with heat-killed but also with living culture of the type 1 strain, T1. Its serum gave a strong precipitin reaction with the homologous M antigen, and regularly protected mice against 1,000,000 M.L.D. of type 1 strains. One portion of serum was absorbed with a known antigenic extract of strain S118 (lot 61, made in the same manner as that used as antigen for the active and passive immunization experiments). Another portion was absorbed with an M extract of strain S118 made with N/20 HCl in a boiling water bath. Neither solution contained demonstrable group-specific C polysaccharide. The optimal proportions point for the precipitation of the serum by each antigen was determined by titration (17), and the solutions of antigens were added to the respective sera in double the optimal proportions. After incubating the mixtures at 37° for 2 hours and keeping them in the ice box overnight, the precipitates were removed and discarded and more of the respective antigen was added to the partially absorbed sera. Practically complete absorption was indicated by the lack of further precipitation with the lot 61 antigen and by the mere trace of precipitate with the M extract prepared by the older method. Serial dilutions of each absorbed serum and of a control lot of the same serum unabsorbed were

TABLE IV

Passive Protection Test in Mice—Type Specificity of Immunity

Serum from rabbit R47-08 immunized with S118 (type 1) extract							
Mice inoculated with test culture							
Dose	Mouse No.	Type 1 strains		Type 3 strains		Type 14 strains	
		S118	T1	D58	C203	S23	T14
cc.							
		(Homologous)					
10^{-3}	1	D 1 day	D 1 day				
	2	D 1 "	D 1 "				
	3	S	S				
	4	S	S				
10^{-4}	1	D 3 days	S				
	2	S	S				
	3	S	S				
	4	S	S				
10^{-5}	1	D 3 days	S	D 1 day	D 1 day	D 1 day	D 1 day
	2	D 4 "	S	D 1 "	D 1 "	D 2 days	D 1 "
	3	S	S	D 1 "	D 1 "	D 4 "	D 8 days
	4	S	S	D 2 days	D 1 "	D 4 "	S
10^{-6}	1	D 2 days	S	D 1 day	D 1 "	D 3 "	D 2 days
	2	S	S	D 1 "	D 1 "	D 3 "	D 4 "
	3	S	S	D 2 days	D 1 "	D 9 "	D 5 "
	4	S	S	D 2 "	D 2 days	S	S
10^{-7}	1	S	S	D 2 "	D 1 day	D 2 days	D 3 days
	2	S	S	D 2 "	D 1 "	D 3 "	D 3 "
	3	S	S	D 3 "	D 2 days	D 4 "	D 6 "
	4	S	S	D 3 "	D 2 "	S	S
Virulence controls: no serum							
10^{-5}	1	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day
	2	D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	S
	3	D 1 "	D 1 "	D 2 days	D 1 "	D 4 days	S
	4	D 1 "	S	D 2 "	D 1 "	S	S
10^{-6}	1	D 1 "	D 1 day	D 1 day	D 1 "	D 1 day	D 1 day
	2	D 2 days	D 1 "	D 2 days	D 1 "	D 1 "	D 1 "
	3	D 2 "	D 1 "	D 2 "	D 1 "	D 2 days	D 2 days
	4	D 2 "	S	D 2 "	D 1 "	S	S
10^{-7}	1	D 1 day	D 3 days	D 1 day	D 1 "	D 1 day	D 1 day
	2	D 2 days	S	D 2 days	D 1 "	D 1 "	D 2 days
	3	D 2 "	S	D 2 "	D 1 "	S	S
	4	S	S	D 2 "	D 10 days	S	S
10^{-8}	1	D 3 days	D 1 day	D 5 "	S	S	S
	2	S	S	S	S	S	S
	3	S	S	S	S	S	S
	4	S	S	S	S	S	S

TABLE IV—*Concluded*

Serum from rabbit R47-23 immunized with D58 (type 3) extract

Mice inoculated with test culture

Dose	Mouse No.	Type 1 strains		Type 3 strains		Type 14 strains	
		S118	T1	D58	C203	S23	T14
cc.							
				(Homologous)			
10^{-1}	1			D 1 day	D 1 day		
	2			D 1 "	D 1 "		
	3				D 1 "		
	4				S		
10^{-2}	1			D 1 "	D 1 day		
	2			D 1 "	D 1 "		
	3			D 1 "	S		
	4			S	S		
10^{-3}	1			S	D 2 days		
	2			S	D 3 "		
	3			S	S		
	4			S	S		
10^{-4}	1			D 1 day	D 1 day		
	2			D 7 days	S		
	3			S	S		
	4			S	S		
10^{-5}	1	D 1 day	D 1 day	S	D 10 days	D 1 day	D 1 day
	2	D 2 days	D 1 "	S	S	D 1 "	D 1 "
	3	D 2 "	D 1 "	S	S	D 2 days	D 1 "
	4	D 2 "	D 1 "	S	S	D 2 "	D 1 "
10^{-6}	1	D 1 day	D 12 days	S	S	D 1 day	D 1 "
	2	D 2 days	S	S	S	D 1 "	D 1 "
	3	S	S	S	S	D 1 "	D 1 "
	4	S	S	S	S	S	D 2 days
10^{-7}	1	D 3 days	S	S	S	D 1 day	D 1 day
	2	D 3 "	S	S	S	D 1 "	D 2 days
	3	D 3 "	S	S	S	D 2 days	D 3 "
	4	S	S	S	S	S	D 5 "

18 to 20 gm. mice were injected intraperitoneally with 0.5 cc. of the serum indicated, the day before inoculation. Fresh 12 hour blood broth cultures were diluted serially with broth so that 0.5 cc. contained the desired dose. The amounts recorded were injected intraperitoneally into four mice in each set. Similar sets of mice which had received no serum were included as virulence controls.

given to mice. On the following day all the mice were inoculated with varying amounts of strain S118.

TABLE V

Precipitin Reactions

Antisera Prepared with Extracts

Serum		M extracts for precipitin tests							Group-specific C fraction
		Type 1 strains		Type 3 strains			Type 14 strains		
		S118	T1	D58	C203	T3*	S23	T14	
	cc.								
Rabbit R47-08 anti-S118 extract (type 1)									
0.2 cc.	0.4	−	++	±	±	+	−	−	−
" "	0.2	−	++	+	+±	+	−	−	−
" "	0.1	+	+	+	±	−	−	−	−
" "	0.05	+±	+±	−	±	−	−	−	−
" "	0.025	+±	++	−	−	−	−	−	−
" "	0.013	+±	++	−	−	−	−	−	−
Rabbit R47-23 anti-D58 extract (type 3)									
0.2 cc.	0.4	++	++	+++	++	++	+	±	−
" "	0.2	+±	+±	+++	+++	+++	+	+	−
" "	0.1	+±	+±	++±	++±	+++	±	+	−
" "	0.05	+	+	+	+±	++	±	−	−
" "	0.025	±	±	±	+	+±	±	−	−
" "	0.013	±	±	±	+	±	−	−	−

The M extracts used here were prepared by heating at 100°C. with N/20 HCl and after alcohol precipitation were made up in a final concentration of about one-fifth of the original volume of extracts. Serial dilutions were made with saline in 0.4 cc. volume. To each tube 0.2 cc. of serum was added, they were then incubated at 37° for 2 hours and kept in the refrigerator overnight before reading. Readings were made on a scale of ++++ to ±. The C fraction used here was made from the type 6 strain S43 by acid extraction and the protein removed with $HgCl_2$. In the concentrations used here it gave strong precipitin reactions with sera potent in anti-C.

Strain S43 was isolated in Texas in 1918 from the throat of a patient with measles.

* Strain T3 is Griffith's strain "Lewis opaque," kindly sent by Dr. Griffith as a representative of type 3. It was given 40 passages through mice to increase its virulence and its content of type-specific substance.

The results of the absorption experiment recorded in Table VI, indicate that the lot 61 extract removed the protective antibody from the antibacterial serum so that even in doses of 0.5 cc. the absorbed serum no longer protected against as little as 100 M.L.D. (10^{-5} cc.) of a strain of homologous type. The absorption with the M substance, extracted at 100° with N/20 HCl, was only slightly less complete: 0.5 cc. of this absorbed serum protected only against 100 and 1,000 M.L.D. and not against larger doses of culture. This amount of protection was insignificant when compared with the original titer of the serum which was high enough for 0.06 cc. to protect a mouse against 100,000 M.L.D.

Active Immunization of Mice with M Extracts

The hypothesis that the M substance is essentially similar to the antigenic substance in extracts active in inducing immunity was also tested by the following experiment.

Mice were immunized actively with M extracts prepared by heating living streptococci with N/20 HCl for 15 minutes in a boiling water bath. The antigen was further purified by reprecipitating twice from saline solution with three to four volumes of 95 per cent ethyl alcohol. A neutral solution of the antigen was filtered through a Berkefeld N filter; and the amount to be injected was calculated on the basis of the trichloroacetic acid precipitable fraction. The antigen was distributed in tubes, each containing enough for one day's immunization, and dried from the frozen state on the Flosdorf-Mudd lyophile apparatus.

In Table VII are recorded the results of testing mice immunized with the extract. One set of animals received 0.33 mg. of material, the same dosage as those recorded in Tables I and II. Only 20 per cent of these survived an inoculation of 100 M.L.D. of the homologous strain S118. In another set which was immunized with 6.6 mg., 60 per cent survived a similar inoculum. A preliminary test with a similar M extract, but unfiltered, also showed 60 per cent survival when large immunizing doses were given for 4 weeks.

Preliminary Chemical Studies of the Antigenic Extract

Total nitrogen and phosphorus analyses on three typical extracts are given in Table VIII. Since the high phosphorus content and the precipitability at pH 4.5 suggested that nucleic acid might be present, spectroscopic examination of several preparations was made.[5] All showed an absorption spectrum characteristic of nucleic acid, that is, a wide band with maximal absorption at about 2,600 Å. Quantitative spectroscopic estimates, using yeast nucleic acid as a standard, indicated that usually 25 to 30 per cent of the material in the extracts was nucleic acid. The biuret test and the Sakaguchi test for arginine were positive in high dilutions of the extracts. Since the relationship

TABLE VI

Absorption Experiment

Passive Protection Test in Mice

Type 1 antibacterial serum		Culture: Type 1, strain S118			
		10^{-2} cc.	10^{-3} cc.	10^{-4} cc.	10^{-5} cc.
	cc.				
Unabsorbed	0.5	S	S	S	S
	0.25	S	S	S	S
	0.12	S	S	S	D 2 days
	0.06	S	D 3 days	D 2 days	D 12 "
	0.03	D 3 days	S	D 2 "	S
Absorbed with type 1, S118 extract: Lot 61 antigen used for active and passive immunization tests	0.5	D 1 day	D 3 days	D 1 day	D 1 day
	0.25	D 1 "	D 2 "	D 2 days	D 2 days
	0.12	D 1 "	D 1 day	D 1 day	D 2 "
	0.06	D 1 "	D 1 "	D 2 days	D 2 "
	0.03	D 1 "	D 1 "	D 2 "	D 1 day
Absorbed with type 1, S118 M extract, made with N/20 HCl at 100°C.	0.5	D 1 "	D 1 "	S	S
	0.25	D 1 "	D 2 days	D 2 days	D 2 days
	0.12	D 1 "	D 1 day	D 2 "	D 5 "
	0.06	D 1 "	D 1 "	D 1 day	D 3 "
	0.03	D 1 "	D 1 "	D 2 days	D 3 "

Virulence controls: Inoculated with strain S118

20 mice inoculated with 10^{-6} cc.	6 mice inoculated with 10^{-7} cc.	6 mice inoculated with 10^{-8} cc.
10 mice D 1 day 8 " D 2 days 2 " S	3 mice D 2 days 1 mouse D 5 " 2 mice S	6 mice S

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[5] We are indebted to Dr. George Lavin for the spectroscopic determinations on these preparations.

TABLE VII

Active Protection Test in Mice
Immunized with M Extract of Strain S118*

Immunised mice: Immunity tested by inoculating with 10^{-6} cc. strain S118

Mouse No.	Each mouse immunized with total dosage 0.33 mg. of extract	Each mouse immunised with total dosage 6.6 mg. of extract
1	D 1 day	D 1 day
2	D 1 "	D 1 "
3	D 1 "	D 2 days
4	D 2 days	D 4 "
5	D 4 "	S
6	D 4 "	S
7	D 4 "	S
8	D 4 "	S
9	S	S
10	S	S

Virulence controls: Inoculated with strain S118

20 mice inoculated with 10^{-6} cc.	5 mice inoculated with 10^{-7} cc.	5 mice inoculated with 10^{-8} cc.
16 mice D 1 day 2 " D 2 days 1 mouse D 5 " 1 " D 12 "	2 mice D 1 day 3 " D 2 days	4 mice D 2 days 1 mouse S

Immunization was carried out in the same manner as with the mice reported in Table I, each set receiving four series of injections.

* Extract prepared by heating at 100°C. with N/20 HCl.

TABLE VIII

Chemical Analysis of Typical Fractions Used for Immunization

Lot No.	Extract from		Organisms heated at 56°C.	N	P	N precipitated by trichloroacetic acid	Nucleic acid
			min.	*per cent*	*per cent*	*per cent*	*per cent*
61	Strain S118	Type 1	15	16.73	3.97	52.06	33.3
62	" D58	" 3	15	15.60	3.69	50.61	6.9
65	" S118	" 1	5	16.25	3.40	58.72	30.2

of these findings to the constitution of the active agent in the extract is not certain, further work is being carried on in an attempt to clarify this point.

DISCUSSION

A substance has been obtained from extracts of group A hemolytic streptococci which induces active immunity in mice. On injection into rabbits it leads to the production of relatively type-specific antibody with which mice may be passively protected against infection with strains of the homologous type. Although the immunity in general was predominantly type-specific in nature, some non-type-specific reactions were also observed. It is important to realize that the methods employed for extracting the streptococci would probably not yield any single substance in a form approaching purity. This conception is strengthened by the previous work which suggests that the hemolytic streptococcus contains many proteins which are precipitable at a pH near 4.5 (2) and this idea is, moreover, confirmed by the cross reactions seen in precipitin tests with some of the antisera obtained from rabbits immunized with this material. Although the first experiment (Table I) indicated active protection against the homologous strain, the second experiment (Table II) showed that this was not strictly type-specific. This cross immunity may be explained by assuming either that it was induced by other antigens in the immunizing extracts having a broader specificity than the type-specific substance or that there are chemical and antigenic relationships among the type-specific substances themselves.

In the passive protection experiments, on the other hand, type-specific immunity was striking, and the indication of cross protection among types so slight that its existence is questionable. In the precipitin tests with the immune sera prepared in rabbits and used for passive protection tests in mice (Table V), both type specificity and cross reaction were observed. The cross reactions here are open to the same interpretation as in the experiment on active immunity. Although precipitin absorptions were not performed in the present experiments, previous work based on absorption experiments indicates that cross reactions observed in the precipitin test are probably due to an admixture of antigens in the extract used to immunize animals, and, correspondingly in the M extract used as reagents in the test tube.

In developing a method of extracting the antigen, different procedures were tried, and the extracts were compared in their ability to produce immunity in mice. While the method used in the present experiments was the best of those tried, there are certain steps in the procedure which were used empirically. In the light of experience gained since this method was adopted, it may be that heat-killing and grinding the bacteria prior to extraction are not essential steps in obtaining the best antigens. We feel that it is important, however, to use young, actively growing cultures, prepared by the method described. The comparative experience in immunizing rabbits, which shows that large doses of antigenic extract did not induce as potent antisera as smaller doses of whole streptococci, makes us feel that the antigenicity of the active substance had been impaired by extraction. Possibly some refinement of these extraction methods or a different procedure may furnish an antigen with unimpaired or little diminished activity.

The nitrogen and phosphorus analyses of the extracts, the positive protein tests, and the spectroscopic analyses, as well as the behavior of the extracts in precipitating at pH 4.5, all suggest the presence of a nucleoprotein. It is impossible to say at present whether a nucleoprotein is the agent active in inducing the immunity observed, since the extracts were undoubtedly impure preparations. Conceivably a very small admixture of some other substance may have been responsible for the antigenic activity.

The antigen we have extracted cannot, as yet, be compared with the labile antigen described by Mudd and his coworkers,[1] since they have studied that fraction mainly in its ability to absorb precipitins, agglutinins, opsonins, and of protective antibodies from antibacterial serum, while we have confined ourselves principally to the production of active immunity in mice and protective antibodies in rabbits. Sevag, Lackman, and Smolens (18) have recently stated that the labile antigen is a nucleoprotein. While the active agent in the extracts described by us may be a nucleoprotein, it does not necessarily follow that it is the same as the labile antigen, since it is known that there are many nucleoproteins in the streptococcus (2), some of which are undoubtedly non-type-specific in nature.

The extract studied by Stamp and Hendry, however, is similar to the one described here, since both are made with N/10 HCl at 37°C. Although the subsequent method of purification is different, both extracts produce active immunity in mice against the homologous strain of streptococcus.

The active protection experiment in mice with the type-specific M substance extracted with N/20 HCl in a boiling water bath, shows that antigenic material is present, even though it is necessary to give larger amounts of it to obtain a degree of protection comparable to that elicited by the extract described in this paper. Both kinds of extracts react similarly in precipitin tests with specific immune sera, and both are also similar in the way they absorb the protective substance from immune sera. This last finding is at variance with the conclusions of Mudd and his collaborators, who were unable to absorb the protective substance from antibacterial sera with M substance prepared as described above.

The evidence presented, together with the general characteristics of the substance and manner of isolation, leads us to believe that the active principle in the extracts used for active and passive immunization in the present experiments is essentially similar to the active principle in the M extracts as formerly prepared, and probably also to the antigenic substances obtained by Stamp and Hendry, although different preparations exhibit varying degrees of purity and degradation from the native state. This substance, immunologically distinct for each serological type of group A hemolytic streptococcus, is probably the type-specific constituent in at least partially antigenic form.

SUMMARY

1. A substance extracted from group A hemolytic streptococcus is described, which induces active immunity in mice, and in rabbits gives rise to precipitins and to protective antibodies passively transferable to mice.

2. The active immunity in mice is principally type-specific, but some degree of non-type-specific immunity is also developed. The passively transferable protective antibodies are type-specific with only a slight suggestion of non-type specificity. In the precipitin test, the rabbit immune sera give both type-specific and non-type-specific reactions which have not been fully analyzed serologically.

3. Substances contained in the extract absorb the protective antibodies from the serum of rabbits immunized with whole hemolytic streptococci.

4. The most satisfactory method of extraction so far developed is fully described. Chemical tests on the material are consistent with the presence of protein and nucleic acid.

5. The type-specific M substance, prepared as previously described, was compared in some of its antigenic properties with the above mentioned substance. It was found capable of inducing active immunity in mice and of absorbing protective antibody from antibacterial immune serum in a manner qualitatively similar to that obtained with the preparations made by the newer methods.

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THE EFFECT OF DIET ON THE PATHOLOGICAL CHANGES IN RATS WITH NEPHROTOXIC NEPHRITIS

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Nephrotoxic nephritis has been regarded by Masugi and the majority of recent workers as the experimental renal lesion which most closely simulates Bright's disease in man.[1,2] Previously we demonstrated that severe acute nephritis induced in rats by means of nephrotoxin generally progressed to a chronic disease with ultimate kidney failure.[2,3] An occasional rat, however, when fed a stock diet, recovered from the acute renal injury, while a number of animals with chronic nephritis of a year's duration still maintained a normal urea clearance value. This variation in the response of individual animals suggested that the course of the disease might be influenced by internal environmental factors, the "milieu intérieur" of Claude Bernard. Since diet has long been considered an important therapeutic measure in the treatment of Bright's disease, and has been shown to affect the clinical picture in one type of experimental renal injury,[4] it seemed desirable to study its influence on the diffuse glomerulonephritis which results from injection of antikidney serum. Accordingly, severe nephrotoxic nephritis was induced in a large group of rats and the course of the renal disease was observed for 10½ months to determine the influence of different diets. Detailed clinical and chemical studies on 48 of the animals in this group are to be reported elsewhere. The present paper deals with the pathological changes found in these rats and in others followed for a shorter period; renal lesions observed in normal rats maintained on one of the experimental diets are also described.

Materials and Methods

Nephrotoxic Serum: Antikidney serum was prepared in rabbits by immunization with sterile suspensions of perfused rat kidney.

Throughout this study a single serum was used, *viz.* that from Rabbit 4557 which was always capable of inducing severe nephritis in rats receiving a total of 0.65 cc. per 100 gm. of body weight, in three divided doses given on consecutive days.[5]

Animals: Young hooded rats of the Whelan strain were injected when they weighed about 100 gm. Males and females were distributed equally in the respective diet groups.

Diet: Rats were fed on one of three isocaloric diets. Each ration contained 27 per cent fat, 4 per cent Osborne and Mendel salt mixture No. 1,* and vitamins plus the following constituents: Diet L—5 per cent protein and 64 per cent carbohydrate; Diet B—18 per cent protein and 51 per cent carbohydrate; Diet H—40 per cent protein and 29 per cent carbohydrate. The protein consisted of Lactalbumin, the fat was chiefly Crisco, while the carbohydrate was composed of a mixture of 2 parts Karo powder and 1 part cane sugar. Cod liver oil U.S.P. which made up 5 per cent by weight of each diet contributed part of the dietary fat and vitamins. Brewers yeast 1 gm. (wet) given on alternate days provided further vitamins. Food and water were always available to the rats.

Technical Procedures: The methods used for collecting clinical and chemical data have been previously described.[3] Moribund animals were generally etherized in order to obtain well preserved tissues. Organs were fixed in Zenker's solution and in 10 per cent neutral formalin. Paraffin sections were stained with eosin and methylene blue, and in addition, Mallory's aniline blue stain and McGregor's[6] modification of the Mallory-Heidenhain method were applied to sections of all kidneys. The scharlach R method was used on frozen sections to show fatty changes. Other staining techniques were occasionally employed.

EXPERIMENTAL

Clinical Course of Nephrotoxic Nephritis in Rats Maintained on Different Diets

The course of the experimental nephritis was similar during the first month in all the rats irrespective of the type of diet that was fed.† Severe albuminuria with cylindruria appeared and persisted. Anasarca was present for a variable number of days. Plasma protein values, which were low during the edematous phase, had generally returned to normal 1 month after the injections of nephrotoxin. On the other hand, blood urea nitrogen values and urea clearance determinations remained within normal limits during this early period, except in a few rats which are not included in this report because they succumbed to the acute disease.

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* Harris product.

The clinical findings in the group of nephritic rats fed Diet L (low protein-high carbohydrate) diverged markedly during the 2nd month from those of the animals fed the other two diets. Seventeen of the 19 animals that received this diet showed either a marked diminution, or, in a few instances, a complete disappearance of urinary abnormalities. Four of these rats were sacrificed at the end of the 3rd month for histological study. Two animals which had excreted urine of a consistently abnormal character died suddenly in the 5th month of the disease without antecedent renal failure. Eight and one-half months after nephritis had been induced, none of the 13 survivors had elevated blood urea or abnormal urea clearance values; moreover, only 1 showed moderate amounts of protein and casts in the urine, 4 had normal urines and the remainder occasionally put forth traces of urinary protein or a few casts. Two of the rats, which had apparently recovered, and 1 of those with slight evidences of nephritis were sacrificed at this time, while 5 of the 10 surviving rats were changed from Diet L to Diet H (high protein-low carbohydrate). During the final 2 months of observation it became apparent that the change to Diet H had had an adverse effect on the diseased kidneys. The single animal in this subgroup with normal urine at 8½ months did not suffer a relapse, but the other rats with low grade or latent nephritis showed an increase in albumin and casts sufficient to warrant a diagnosis of mild or moderate kidney irritation. Exacerbations of this sort did not occur in the rats maintained on the original Diet L.

Every animal fed Diet H (high protein-low carbohydrate) after receiving antikidney serum continued to have marked albuminuria and cylindruria until it died or was sacrificed. Four members of this group were sacrificed 3 months after injection. Only 2 of the 15 remaining rats survived until the experiment was terminated 10½ months after induction of nephritis; moreover, both of these animals were in a terminal phase of chronic progressive nephritis. Among 13 rats dying with renal failure, the average time of survival after injection was 6 months.

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† Data presented in abstract, Farr, L. E., and Smadel, J. E., *Proc. Soc. Exper. Biol. & Med.*, 1937, **36**, 472–473.

The course of the disease in rats fed on Diet B (basal) was similar to that observed in earlier experiments when the animals were maintained on a varied stock laboratory diet.[3] One of the 15 rats in this group recovered clinically during the 2nd month, while 6 showed abnormal urinary contents throughout the experiment but did not develop renal failure. Eight of the 15 rats died of kidney insufficiency; their average time of survival was 5½ months.

Smaller groups of normal rats fed on Diets L and H for several months were sacrificed for histological study. These animals excreted normal urine throughout the period.

Histopathological Observations

The characteristic lesions in rats with acute nephrotoxic nephritis and the chronic nephritis that follows the acute injury have been described.[3] Both glomeruli and tubules are affected in the acute disease. The tubular epithelium shows necrobiotic changes, principally hyaline droplet degeneration, while the outstanding glomerular lesion consists of swelling of the glomerular capillary basement membranes.

Renal Lesions in Rats Fed Diet B

The nephritic animals maintained on this normal basal diet not only followed the same general clinical course as those kept on a stock diet in previous experiments, but also had essentially the same type of kidney damage; hence, only a summary need be given here. Rats that died of progressive nephritis from 68 to 237 days after receiving nephrotoxin had enlarged kidneys with granular surfaces, and many cystic dilated tubules filled with coagulated material were visible macroscopically throughout the cut sections, especially in the corticomedullary region. These changes were most marked in animals with long-standing disease. Microscopic examination showed that practically all the glomeruli were abnormal: those least affected had distorted tufts with irregularly thickened glomerular capillary basement membranes; in others the epithelium of Bowman's capsule was proliferated and the capsular membrane was thickened, and there were also glomeruli with varying amounts of connective tissue replacement. Glomerular changes of differing degree were always demonstrable in each section, although the number of severely scarred glomeruli was increased in rats with a more chronic disease. Severe damage was found in tubular structures on histological study; extensive dilatation with large hyaline casts, atrophy in areas of interstitial scarring, and epithelial hyperplasia of the remaining functioning units were all observed. The epithelium of the functioning tubules showed various grades of degeneration, including necrosis, in animals killed when moribund. Vascular lesions as well as perivascular and interstitial infiltrations of cells were characteristically present. Rats in this group, which survived the period of the experiment without developing renal failure, even though showing evidence of chronic nephritis until sacrificed, had the same types of kidney lesions, but many functioning renal units remained.

The single animal in this group in which the nephritic process diminished markedly during the 2nd month, and thereafter became negligible, had kidneys with surfaces that were essentially smooth. Moreover, on microscopic examination most of the structural units appeared normal. A rare dilated tubule filled with a hyaline cast, and an occasional small, completely scarred glomerulus could be found. However, fair numbers of glomeruli which appeared capable of functioning, had slight abnormalities in structure. Changes such as irregular thickening of the capillary basement membrane, local dilatation of capillary loops, thickening of capsular basement membrane and, rarely, small crescents were encountered.

The typical macroscopic appearance of a kidney from a rat with progressive nephritis in this diet group is illustrated in Figure 2. Figures 5 and 7 depict characteristic microscopic lesions seen in such kidneys.

Renal Lesions in Rats Fed Diet H

Young normal and nephritic rats thrived when fed the high protein-low carbohydrate diet H; they gained weight faster and grew larger than rats kept on the basal diet B. A normal male animal weighing

90 to 100 gm. when placed on Diet H usually doubled its weight in 2½ months. The kidneys of such an uninjected control rat weighed 1.3 to 1.4 gm. each and, except for slight hypertrophy, were normal on inspection and on microscopic examination.

In general, similar kidney lesions were found in rats with chronic progressive nephritis maintained on either Diet B or H; certain differences, however, usually made it possible to distinguish between members of the two groups on macroscopic evidence alone. The kidneys of rats in the Diet H group were usually smaller. Thus, the average weight of the left kidneys of 7 rats dying in Group B was 1.6 gm., while the average of 13 rats dying in Group H was 1.2 gm. The cortical surface was more coarsely granular in Group H. Finally, the cut sections of kidneys from rats in Group B contained many more cystic tubules filled with hyaline material than did kidneys of animals in Group H. These differences were more pronounced in rats which succumbed 5 to 7 months after injection but are clearly depicted in the two kidneys obtained at the end of the experiment and illustrated in Figures 2 and 3.

All gradations of glomerular change described in rats of Group B were observed in animals fed the high protein-low carbohydrate diet; however, the number of completely or extensively scarred glomeruli was always greater in the latter group of animals for any given duration of the nephritis.

A more striking difference between the two groups was found in the varying proportions of the several types of tubular lesions. Cystic tubules filled with hyaline material, observed in such abundance throughout the cortex and medulla of rats with progressive nephritis in Group B, were less frequent and smaller in rats of Group H. On the other hand, necrobiotic changes in the tubular epithelium were more striking in sections from Group H; moreover, degeneration was observed in cells of the proximal segment of tubules in animals of Group H which were killed (3 months after nephritis had been induced) before renal failure was imminent. Widespread interstitial fibrosis encompassing destroyed tubules was marked in the kidneys of the rats in Group H and was observed as early as 3 months after injection of antikidney serum. In addition, hypertrophy and hyperplasia of the epithelium of remaining functioning tubules were also significantly greater in rats of Group H. Urochrome pigment was conspicuous in the tubular epithelium of rats with terminal anemia irrespective of the diet group. Vascular lesions and perivascular cellular infiltrations occurred with equal frequency and intensity in kidneys of rats that died in the two groups.

In general, glomeruli and tubules which had been damaged by nephrotoxin apparently were unable to undergo repair when the rats were fed the high protein-low carbohydrate diet. Instead, progressive destruction of kidney substance, with connective tissue replacement, proceeded at a more relentless pace than in rats fed the basal diet. Macroscopic and microscopic changes characteristically found in nephritic rats which died while being fed the H Diet are represented in Figures 3, 6 and 8.

Renal Lesions in Rats Fed Diet L

Diet L was sufficiently well balanced to sustain life and to permit a retarded growth of rats, but various abnormalities were observed in animals maintained on it. For example, both untreated and nephritic rats fed Diet L failed to attain normal weight or stature; their fur continued to be soft and short even after the adult state had been reached; and finally, renal and hepatic lesions were consistently observed. It is necessary to present a description of the characteristic pathological changes found in young normal rats maintained on this diet for several months before attempting to evaluate the lesions attributable to nephrotoxin.

A young female rat (PN–13), weight 66 gm., was fed a stock diet for 2 weeks while repeated urine specimens were obtained; no urinary albumin, blood or casts were demonstrable. The animal was then transferred to Diet L. During the next 2½ months this animal gained from 100 to 130 gm. and continued to excrete normal urine.

The kidney, examined at the end of the period, weighed 0.6 gm. and had scattered shallow depressed scars on the cortical surface. On cut section, the corticomedullary region showed numerous, slightly raised yellow streaks arranged in parallel lines radial to the pelvis (see Fig. 11).

The principal changes found on microscopic examination of this kidney occurred in the areas represented macroscopically by the yellow streaks. Groups of moderately dilated tubules were lined by low cuboidal epithelium, often so thinned as to resemble the connective tissue cells which lay outside the thickened and hyalinized tubular basement membrane. The lumens of dilated tubules generally contained refractile material, sometimes arranged in concentric rings, which stained very faintly with the acid dyes and failed to stain differentially with scharlach R or iodine. Remnants of a brush border could still be identified in certain of the affected proximal convoluted tubules. In the same area, atrophic tubules lined by epithelium with basophilic cytoplasm and with collapsed lumens were present. Their basement membranes were also hyalinized and surrounded by scar tissue. Less drastic changes were demonstrable in other proximal tubules of the corticomedullary area. In some, only a single epithelial cell was degenerated or necrotic, while in others many were affected and cast off cells lay loose in the lumen. Fat was often demonstrated in such damaged cells by scharlach R stains. Regeneration of epithelium was observed and occasionally resulted in an irregular stratification of cells protruding into the lumen. Thickening of the tubular basement membrane or increase in interstitial connective tissue was not conspicuous here. Figure 12 illustrates the microscopic findings in the corticomedullary region of this kidney.

Most of the glomeruli appeared normal but a few had changes in the tuft and occasionally extensive scarring was observed. Damaged glomeruli were usually found near diseased tubules and occasionally could be shown to connect with one of them. Vascular lesions were not found, and interstitial collections of lymphocytes, when present, were small and limited to the corticomedullary scars.

An extreme fatty change was present throughout the liver. The least affected parenchymatous cells, about the portal spaces, contained fat globules, while in the central portion of the lobules the cytoplasm of individual cells stained a homogeneous red with scharlach R.

The renal and hepatic injury which occurred in all young rats fed Diet L for several months varied in degree. Rat PN–13, described above, was one of the most severely affected of the group. On the other hand, Rat PN–7, which seemed to recover from nephrotoxic nephritis, had a minimal amount of the kidney damage attributable to the low protein-high carbohydrate diet when sacrificed 3 months after injection of antikidney serum.

A young male rat (PN–7) weighed 55 gm. when nephrotoxin was administered and Diet L was started. The acute nephritis began to subside in several weeks and the urine was normal 4 weeks after nephritis had been induced. The animal weighed 94 gm. when sacrificed 3 months after injection. The kidneys, which weighed 0.5 gm. each, were apparently normal on macroscopic examination but showed glomerular lesions throughout the microscopic section. Practically all of the tuft capillaries contained blood but their walls were significantly thickened by an increased width of the basement membranes. About 20 per cent of the tufts were lobulated or otherwise distorted, and approximately 10 per cent of them were adherent to the capsular epithelium in one or more places; nevertheless, well developed crescents were not seen. A few tufts contained scattered cells, apparently epithelial in origin, with large, bright eosin-staining granules in their cytoplasm. Scattered small areas in the corticomedullary region contained dilated tubules, some of which were filled with laminated pale staining material similar to that seen in Rat PN–13; a few atrophic tubules were also present in these areas. An occasional atrophic tubular structure in the cortex was seemingly related to the pathological areas at the junction of cortex and medulla. In addition, tubules containing hyaline casts were occasionally encountered. Vascular lesions and interstitial cellular infiltrations were not observed.

The liver was the seat of mild fatty change. Microscopic sections of the heart were normal.

The kidneys of rats fed Diet L for 8 to 10½ months after nephritis had been induced with nephrotoxin were similar to those of members of the group sacrificed at 3 months. The lesions attributed to the diet were, however, less obvious at 10½ months. The kidneys of only a few of the animals had macroscopic yellow streaks in the corticomedullary region, which on microscopic examination appeared to be dilated tubules filled with poorly staining material. Narrow bands of old connective tissue arranged radially were present in this portion of all the kidneys; these were often conspicuous but sometimes appeared only once or twice in the entire section. Five to 10 per cent of the glomeruli in the kidneys from older animals were represented by small contracted scars; in addition a few had moderate hyperplasia of the epithelium of Bowman's capsule. The majority of glomeruli appeared to be functioning but showed abnormalities of the tufts such as described in Rat PN-7. The capillaries of the tufts were often dilated in places and contained puddles of blood; these frequently occurred at the border of the tuft. Scattered atrophic tubules were present throughout the cortex. Vascular lesions and interstitial cellular infiltrations were not present in the kidneys of the group of older rats, nor were generalized vascular changes observed.

The livers of the animals sacrificed at the later date showed about as much fatty change as did those examined earlier; certain of the rats also had cirrhosis.

There was no consistent histological difference detected between the kidneys of rats maintained on Diet L throughout the experiments and those of rats transferred from Diet L to Diet H 8½ months after injection of antikidney serum. Fatty change in the livers of rats in this last group, however, was slight when present.

Two rats, in Diet Group L, failed to make a clinical recovery during the 2nd month and died 134 and 139 days, respectively, after nephritis had been induced. Neither of these animals had a significant reduction of kidney function at any time, but their urine contained 1 to 3 gm. of protein per 100 cc. and numerous casts until the end. Kidney lesions in these 2 rats were more extensive than in other members of the group but hardly severe enough to account for the early death. Changes in the tufts, crescent formation, complete scarring of glomeruli, numerous dilated tubules filled with hyaline casts and degenerative changes in tubular epithelium suggested the picture seen in nephritic rats fed Diet B. Both livers were heavily laden with fat. The death of these 2 animals probably depended on the combined effect of severe chronic nephritis and poor diet.

These histopathological observations agree with clinical data and indicate that rats with severe acute nephrotoxic nephritis tend to recover when maintained on a low protein-high carbohydrate diet. The tubular changes characteristic of the acute nephritis appear to be reversible for the most part when circumstances are propitious. Glomerular lesions, on the contrary, do not disappear; but notwithstanding the presence of residual changes, most of the glomeruli continue to function.

Hematuria in Rats with Chronic and Latent Nephritis

Pearce[7] held that hematuria was not a result of injury with nephrotoxin but depended on other factors in antikidney serum. We have also emphasized this point in reference to the acute syndrome induced by nephrotoxin.[3] Present observations offer no reason for changing our views on hematuria in the acute nephritis but do indicate that renal bleeding may occur in rats after the acute phase of renal injury has passed.

Hematuria, observed in 7 rats, was generally of the intermittent type and when it occurred was obvious macroscopically. Two rats in Group L showed red cells in the urine for the first time after they had been fed the low protein-high carbohydrate diet for 9 and 10 months, respectively; moreover, there had been neither casts nor more than a trace of albumin for months before the appearance of blood in the urine. Onset of hematuria in the 4 rats of Group B occurred in the 3rd (2 animals), 4th and 7th months and, in a single animal of Group H, hemorrhage was detected during the 7th month on one occasion.

Hematuria in these animals seemed to have no adverse prognostic significance for all of the rats lived throughout the experiment. No evidence of an infectious process which might have accounted for the bleeding was found in any of the kidneys, and cultures of renal tissue made at the time of autopsy were bacteriologically sterile. Dilated capillaries near the margins of diseased glomerular tufts were observed in rats of all three diet groups, and rupture of such diseased capillaries seems a likely explanation for the intermittent hematuria.

Generalized Vascular Lesions in Rats with Chronic Progressive Nephritis

Thickening and hyalinization of the walls of small arteries and of arterioles occurring in various organs of rats which succumbed to chronic progressive nephritis were described and illustrated in an earlier report.[3] Additional vascular changes, such as the presence of fat or of calcium in the media and a reduplication of the internal elastic membrane of coronary arteries, and perivascular cellular collections in the pancreas were recorded. The occurrence of chronic generalized vascular lesions has also been noted in the present group

TABLE I

Extrarenal Vascular Lesions in Rats That Succumbed to Nephritis

Diet (protein)	Number of rats	Average time of survival	Heart			Pancreas			Brain			Intestine			Liver			Testicle		
			A	C	T	A	C	T	A	C	T	A	C	T	A	C	T	A	C	T
L (low)	2*	4½ mo.			0/2			0/0			0/2			0/0			0/2			0/0
B (basal)	7	5½ mo.	2/7	6/7	7/7	1/4	4/4	4/4	0/5	4/5	4/5	1/1	0/1	1/1			0/7			0/2
H (high)	13	6 mo.	3/13	7/13	9/13	0/9	2/9	2/9	0/12	2/12	2/12	1/1	0/1	1/1	0/11	6/11	6/11	0/7	3/7	3/7

A = Acute lesions

C = Chronic lesions

T = Total number of rats in each group showing lesions of acute or chronic variety or both

The numerator of each fraction indicates the number of rats with lesions; the denominator the number of organs examined.

* Rats did not have renal failure immediately before death but had severe chronic nephritis. Death probably resulted from a combination of nephritis and poor diet.

of rats which died after long-standing nephritis. Their distribution is enumerated in Table I.

Fibrous myocarditis and encephalomalacia which apparently depended on these chronic vascular lesions were found in 13 of 22 rats with chronic nephritis terminating in death; myocardial scarring occurred 5 times in Group B and 3 times in Group H, while encephalomalacia was noted in 3 members of the former group and in 2 of the latter.

In addition to the chronic extrarenal vascular lesions, other changes of a more acute nature were observed, especially in the heart and intestine. These consisted of proliferation and swelling of endothelium, fibrinoid degeneration in the muscle coat, and pyknosis of muscle nuclei. Table I records the frequency of acute vascular lesions such as are illustrated in Figures 13 and 17.

Vessel changes in the heart, brain and pancreas, as well as myocarditis, were found in 1 of the animals of Group B which survived to the end of the experiment; however, its renal function had been depressed for a month before it was sacrificed. Renal insufficiency was present in both rats of Group H when they were sacrificed 10½ months after injection of nephrotoxin. One had vascular lesions in the heart, brain and testicle and also myocarditis, the other had only coronary abnormalities. None of the rats in Group L which were sacrificed during the experiment, or at its end, had generalized vascular lesions.

Acute Myocarditis and Enteritis in Rats with Renal Failure

An acute focal necrosis of cardiac muscle fibers was frequently seen in rats dying with chronic renal insufficiency. These lesions could usually be identified macroscopically as yellow flecks beneath the epicardium and endocardium. On microscopic examination some groups of degenerated cardiac muscle cells were represented by necrotic débris while others still retained their general outline. The cellular reaction in and about such areas was in the majority of cases mononuclear in type but occasionally consisted of polymorphonuclears or, rarely, was entirely absent. In certain areas cellular collections surrounded muscle fibers which retained their striations. Large mononuclear cells were the principal elements in these collections, but an occasional multinucleated cell was seen. Acute lesions, when present, were usually numerous; one section contained nine foci. The myocardium pictured in Figure 16 contained many affected areas. Acute myocarditis occurred in 5 of the 7 rats in Group B and in 9 of the 13 animals in Group H that died with progressive nephritis. Three of the former animals and 2 of the latter had, in addition, old scarring of the myocardium.

Fibrinoid swelling of the ground substance was found in 4 hearts which contained other acute lesions; 2 rats each were involved in Groups B and H. An area of intense fibrinoid degeneration is shown in the left lower portion of Figure 16, while a milder type of reaction is discernible in the area of focal myocarditis illustrated in Figure 15.

Acute myocardial lesions occurred in 14 of the 20 rats with progressive nephritis and protracted renal insufficiency terminating in death. On the contrary, this type of cardiac involvement was not observed in rats of Groups B and H that survived throughout the experiment even though some of these animals showed lowered urea clearance values during the later months of observation; moreover no such lesions were detected in any rat of Group L. McJunkin *et al.*[8] described similar foci of acute myocardial necrosis in nephrectomized rats which were injected with large amounts of phosphate. Those authors do not, however, emphasize the occurrence of swelling and proliferation of endothelium in small coronary arteries and capillaries and these changes, which were usually prominent in our rats with acute myocarditis, may have played some part in the formation of foci of necrosis.

Typhlitis was a contributing cause of death in 2 animals with renal failure; 1 in Group B died on the 198th day and the other in Group H survived 136 days after nephritis was induced. In both instances the cecum showed macroscopic subserosal ecchymoses, edema and hemorrhage of the wall, pseudomembranous exudate covering the mucosa, and blood in the lumen. Figure 14 illustrates such a lesion. Vascular changes of the acute variety were conspicuous near the border of the involved areas (see Fig. 13) and probably were responsible for the intestinal lesion. Goldblatt[9] has recently reported that intestinal bleeding is one of the cardinal signs of the malignant phase of hypertension in dogs. While blood pressure readings were not made in the present experiment, hypertension was previously demonstrated in rats with chronic progressive nephritis of nephrotoxic origin.[3]

DISCUSSION

The experiments here presented demonstrate that the course of the nephritis in rats which follows injection of antikidney serum is significantly influenced by at least one internal environmental factor, namely, diet. Clinical evidence of renal disease rapidly subsided in almost all young rats placed on a low protein-high carbohydrate diet after severe nephrotoxic nephritis had been induced. Moreover, the acute tubular injury resolved without permanent damage and only moderate residual changes were present in the glomeruli. Renal failure generally occurred within a year, when nephritic rats were maintained on a high protein-low carbohydrate diet, and progressive destruction and scarring of both tubules and glomeruli was observed histologically. More than half the nephritic rats fed a basal diet developed kidney failure and these also had histological lesions indicative of progressive disease. The clinical and pathological features of the nephrotoxic disease were the same in rats nourished with this last diet as in rats fed a mixed stock diet.

While the glomerular lesions found in the fatal cases of nephritis in animals fed either the high protein or basal diets were essentially alike, the tubular changes differed in certain important respects. The marked dilatation of proximal tubular segments in kidneys of Group B rats suggests that the nephron had been rendered functionless through occlusion of the loop or of the distal tubule by scarring or plugging. Degenerative changes were found in the proximal tubular epithelium of kidneys in Group H even when the animals were sacrificed relatively early in the disease; and, in addition, obliterative scarring of proximal tubules was characteristically present in kidneys of the rats that died. It thus appears that one of the most significant effects of diet on the course of nephrotoxic nephritis involves the response of the acutely damaged tubular epithelium. There was a tendency towards recovery from tubular injury when a low protein-high carbohydrate diet was fed, while progressive involvement of the distal tubular segment was characteristically found when a basal diet was given, and, finally, destruction of the proximal tubule was outstanding in nephritic rats maintained on a high protein-low carbohydrate diet. The studies of Richards and Walker[10] on selective action of different portions of the kidney tubule in frogs and necturi suggest an approach to an explanation of our observations in rats, but the exact factors responsible for the recovery of the nephron or destruction of its proximal or distal segment are not clear. The response of the glomerular capsular epithelium was similar to that observed in the remainder of the nephron. Obliteration of the capsular space by crescent formation occurred relatively infrequently in rats of Group L notwithstanding the fact that residual changes in the glomerular capillary bed were common. In contrast to this, marked proliferation of the epithelium of Bowman's capsule with connective tissue ingrowth into the glomerular tuft was conspicuous in the kidneys of rats in both Groups B and H.

Medlar and Blatherwick[11] have commented on the similarity of the renal picture in chronic dietary nephritis observed in rats with unilateral nephrectomy which were maintained on rations rich in animal protein, and the terminal phase of nephrotoxic nephritis in our rats fed a mixed stock diet. The increased susceptibility of male rats to dietary nephritis noted by these workers was less clear-cut in the present experiments; nevertheless 5 of the 7 rats in Group B and both of the animals in Group H that survived the experiment were females.

Generalized vascular changes of both acute and chronic varieties and the visceral lesions that stem from them were encountered only in rats which developed chronic progressive nephritis and renal insufficiency. Diet apparently influenced production of the extrarenal disease only indirectly by way of its effect on the injured kidney. Differences in the frequency of vascular changes were observed in Diet groups B and H, but probably were not significant. Animals that lived throughout the experiment were approximately 380 days old when sacrificed. The failure to find chronic vascular disease or fibrous myocarditis in our surviving animals with normal urea clearance values agrees with the experience of Wilens and Sproul.[12,13] These authors observed cardiovascular lesions in more than half the members of a large group of senile rats. However they state regarding the heart that "almost all of the changes described make their appearance late in the second year of life and do not attain their maximum incidence until well into the third year." Furthermore, they observed few vascular lesions before the 700th day of life. It may well be that the process of aging and the concomitant development of chronic cardiovascular disease are merely accelerated in rats with severe nephritis.

It should be emphasized that rats used in these studies were all of a black and white hooded strain designated as Whelan stock. Experiments to be reported by Swift and Smadel have indicated that rats of the Wistar and Evans strains respond somewhat differently to the effect of nephrotoxin and diet.

CONCLUSION

In rats of the so-called Whelan strain the chronic nephritis which follows the administration of antikidney serum can be markedly influenced by isocaloric diets containing different proportions of protein and carbohydrate.

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DESCRIPTION OF PLATES

PLATE 38

FIG. 1. Cortical and cut surfaces of kidney from Rat 4 L. Rapid recovery from acute nephrotoxic nephritis occurred on the low protein diet; albuminuria had diminished to a faint trace and casts were absent a month after injection of antikidney serum. Autopsied 10½ months after onset of acute nephritis. Left kidney, weight 1 gm. × 3.

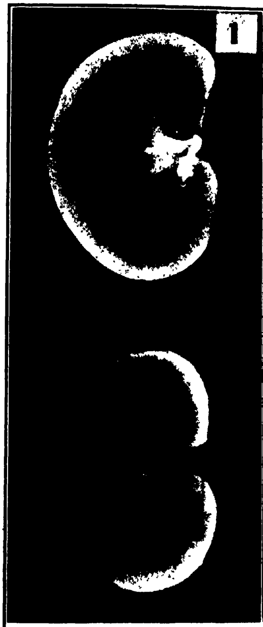
FIG. 2. Kidney of Rat 8 B in Diet group B. Severe albuminuria and cylindruria continued from the time of induction of acute nephritis until the rat was sacrificed 10½ months later; final urea clearance value was within the lower limits of normal. Kidney weight 2.1 gm. × 3.

FIG. 3. Kidney of Rat 3 H fed the high protein diet for 10½ months after injection of nephrotoxic serum. Severe nephritis was evident throughout and a depressed urea clearance was observed during the 2 months preceding sacrifice. Kidney weight 1.7 gm. × 3.

FIG. 4. Rat 4 L. Few abnormalities are detectable in the section of kidney at this magnification. Mallory's aniline blue stain. × 35.

FIG. 5. Rat 11 B, basal diet group, died with renal failure 7 months after onset of nephritis. Extensive destruction of tubules and distortion of glomeruli are evident, but the most conspicuous abnormality is the markedly dilated tubules filled with hyaline material. Mallory's aniline blue stain. × 35.

FIG. 6. Rat 5 H, high protein diet, succumbed to kidney insufficiency 5½ months after receiving nephrotoxin. Glomerular damage is similar to that found in Fig. 5. Nests of hypertrophic tubules constitute the bulk of the substance. Extensive areas of interstitial scarring have replaced destroyed tubules. Mallory's aniline blue stain. × 35.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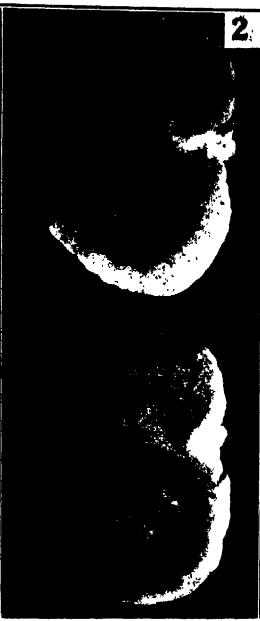
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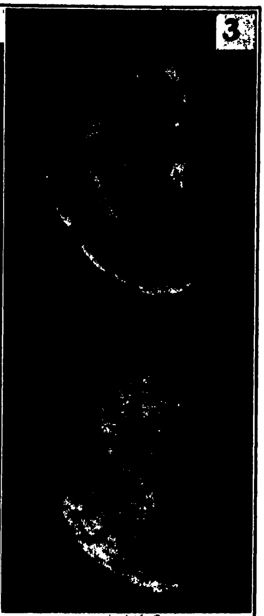
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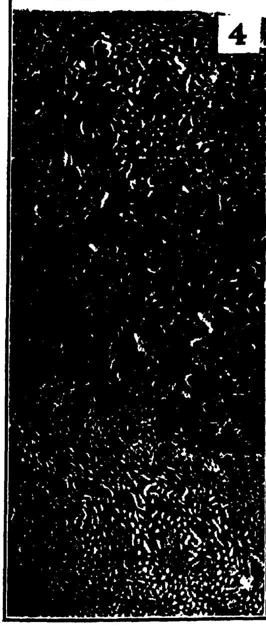
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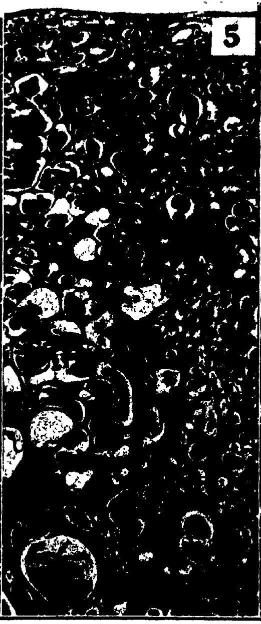
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Smadel and Farr

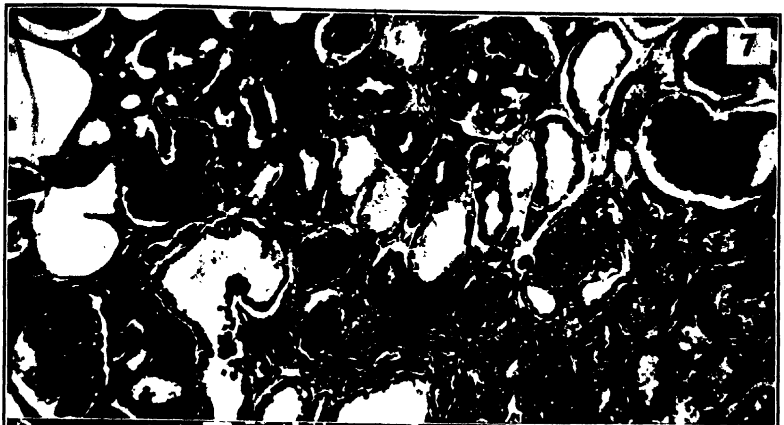
Effect of Diet on Rats with Nephritis

Plate 39

Figs. 7 and 8. Higher magnification of the same sections illustrated in Figs. 5 and 6 respectively. × 150.

Fig. 9. A higher magnification of the section shown in Fig. 4. The tubules are not significantly altered and the glomeruli appear capable of functioning. Moderate irregular thickening of the glomerular capillary basement membranes is apparent. Some distortion of the glomerular tuft on the right is to be seen, while slight proliferation of the capsular epithelium and a small capsular adhesion can be observed in the glomerulus on the left. × 175.

Fig. 10. An area of encephalomalacia in the cortex of the parietal lobe of Rat 13 B, basal diet group, which died of renal failure 5½ months after nephritis had been induced. Eosin-methylene blue stain. × 70.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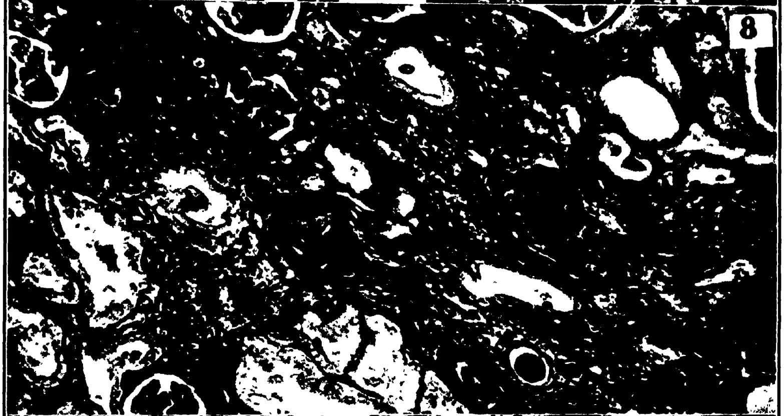
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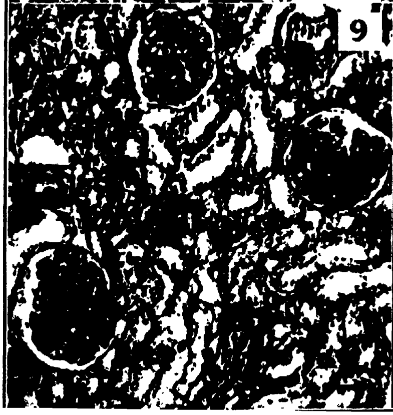
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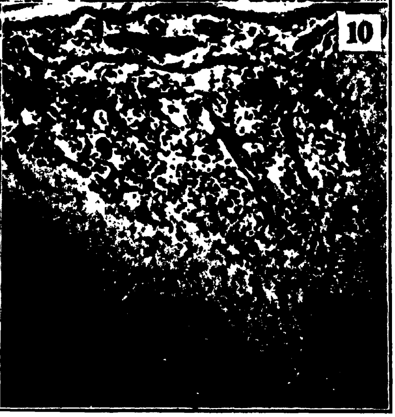
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PLATE 40

FIG. 11. Kidney of Rat PN 13, an uninoculated control animal which was fed the low protein diet for 3 months. The pale streaks in the corticomedullary region were yellow in the fresh specimen. × 4.

FIG. 12. Higher magnification of Fig. 11, showing degenerated tubules with casts in the corticomedullary region. Eosin-methylene blue stain. × 250.

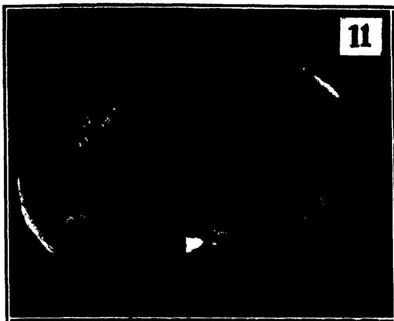
FIG. 13. Section of the cecum of Rat 8 H, which was fed the high protein diet and which died 7 months after the injection of nephrotoxin. An acute necrotizing process is present in all three vessels. These vessels can also be seen at the extreme right in Fig. 14. Eosin-methylene blue stain. × 210.

FIG. 14. Same as Fig. 13. Hemorrhage and edema in the submucosa with a fibrinous exudate covering the necrotic mucosa are shown. × 30.

FIG. 15. Section of the heart of Rat 15 B, basal diet group, which succumbed with nephritis in the 7th month. A small area of focal necrosis of muscle fibers and infiltration with mononuclear cells is seen. Eosin-methylene blue stain. × 450.

FIG. 16. Same as Fig. 15. Multiple focal lesions. An area of fibrinoid change surrounded by muscle fibers showing fresh necrosis is present at the lower left. Areas of necrosis with cellular infiltrations are shown at the lower right and upper portions of the microphotograph. Thickening of vessel walls was also present in other portions of the section. Eosin-methylene blue stain. × 175.

FIG. 17. Heart of Rat 10 B, basal diet group, which died with nephritis after 68 days. Swelling and vacuolation of cells of the media are prominent in the large vessel on the left. Hyperchromatic enlarged cells are seen in the wall of the small vessel in the lower center as well as swelling of the intimal cells. The vessel at the lower right is completely occluded by intimal proliferation. Eosin-methylene blue stain. × 450.



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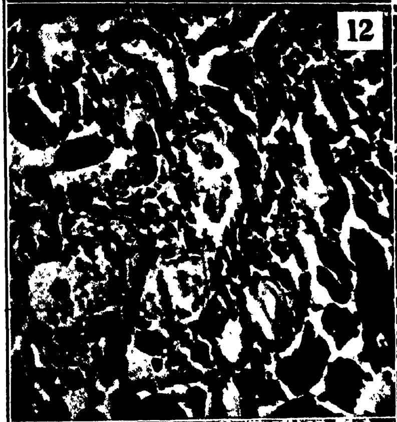

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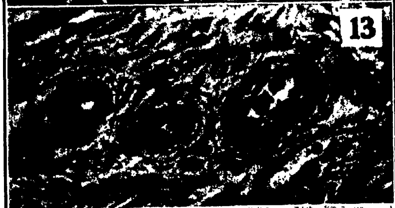

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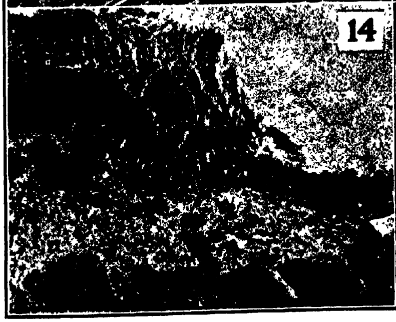

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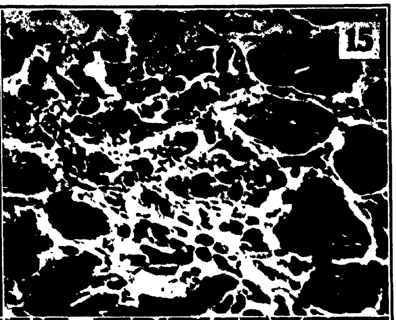

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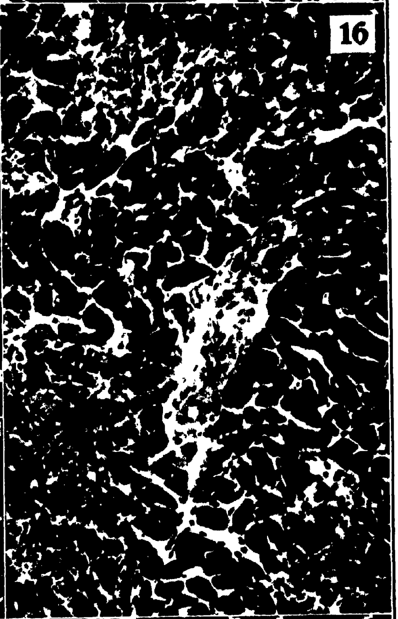

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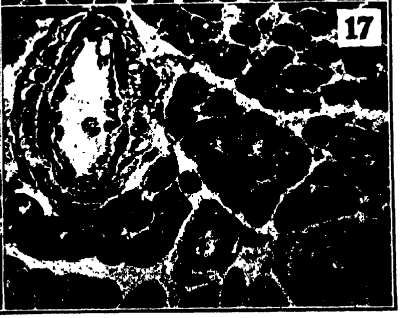

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Smadel and Farr

Effect of Diet on Rats with Nephritis

[Reprinted from the AMERICAN JOURNAL OF DISEASES OF CHILDREN, February, 1939, Vol. 57, No. 2, pp. 306-308]

RELATION BETWEEN PLASMA PROTEIN LEVEL AND EDEMA IN NEPHROTIC CHILDREN

BY LEE E. FARR, M.D., AND DONALD D. VAN SLYKE, PH.D.

(*From the Hospital of The Rockefeller Institute for Medical Research*)

In 1930 Moore and Van Slyke[1] presented data showing the relation between the plasma protein level and the occurrence of edema in adults with nephritis. Subsequently it was reported by Cowie, Jarvis and Cooperstock,[2] in 1930, by Calvin and Goldberg,[3] in 1931, and by Mitchell, Rittershofer, Wang, Kaucher, Wing and Hogden,[4] in 1938, that in children a somewhat lower protein level is compatible with freedom from edema; but no precise levels were given. In the present series of observations, the relation is shown between the level of the plasma protein, particularly the albumin fraction, and the presence of edema in children with the nephrotic syndrome.

Epstein's view[5] has attained general acceptance; he stated that lowering of protein osmotic pressure in the plasma is the chief cause of

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1. Moore, N. S., and Van Slyke, D. D.: The Relationships Between Plasma Specific Gravity, Plasma Protein Content and Edema in Nephritis, J. Clin. Investigation 8:337 (April) 1930.

2. Cowie, D. M.; Jarvis, K. M., and Cooperstock, M.: Metabolism Studies in Nephrosis with Special Reference to the Relationship of Protein Intake to Nitrogen Retention, Edema and Albuminuria, Am. J. Dis. Child. 40:465 (Sept.) 1930.

3. Calvin, J. K., and Goldberg, A. H.: Plasma Proteins and Edema and Their Relationship in a Group of Children Presenting the Nephrotic Syndrome, Am. J. Dis. Child. 42:314 (Aug.) 1931.

4. Mitchell, A. G.; Rittershofer, C. R.; Wang, C. C.; Kaucher, M.; Wing, M., and Hogden, C.: A Metabolic Study of Five Children with the Nephrotic Syndrome: I. A Report on Certain Constituents of the Blood, the Basal Metabolism and the Energy Exchange, Am. J. Dis. Child. 55:27 (Jan.) 1938.

5. Epstein, A. A.: Concerning the Causation of Edema in Chronic Parenchymatous Nephritis: Method for Its Alleviation, Am. J. M. Sc. 154:638 (Oct.) 1917.

nephrotic edema. Observation of the plasma protein level is important in assisting one to predict the degree of difficulty that will be encountered in eliminating edema. The data in the present paper, it is believed, may add to the accuracy of such predictions for children.

One hundred and fourteen observations were made on 10 children of various ages who had edema of the nephrotic type. The duration of illness when the patients were admitted to the hospital varied from three days to three years. Only data are presented which were obtained when the children were free from evident acute infection. The periods over which these data were obtained for given patients varied from nine months to three years. More complete clinical data on some of these patients have been reported by Page and Farr[6] and by Farr.[7]

Restriction of salt was adhered to throughout these periods. The blood to be analyzed was drawn from the veins of the arm in the course of observation and treatment. Oxalated plasma was used for all analyses. The levels of total plasma protein and plasma albumin were determined by Howe's method,[8] the determinations of the nitrogen content being made with Van Slyke's gasometric micro-Kjeldahl procedure.[9] The globulin level was calculated by difference.

RESULTS

The results are summarized in the figure. The regularity with which edema was absent when the plasma albumin level was above the critical point of 1.2 Gm. per hundred cubic centimeters is striking. This critical level for children is markedly lower than the critical level of 2.5 Gm. reported for adults by Moore and Van Slyke. In a small child the albumin content must fall lower than in an adult before persistent nephrotic edema results. The age at which the difference becomes evident cannot be stated, since most of our patients were from 4 to 7 years old.

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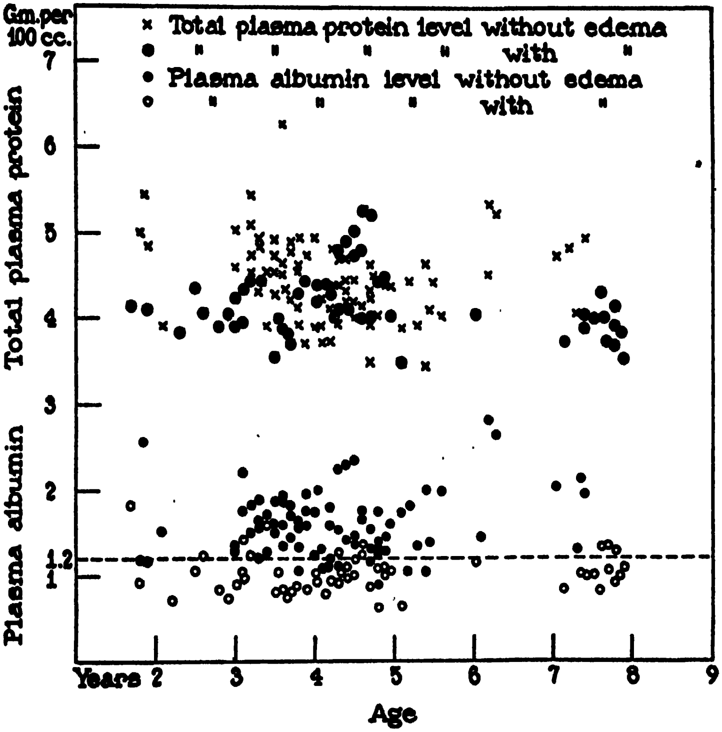
[6] Page, I. H., and Farr, L. E.: The Influence of High and Low Fat Diets and Thyroid Substance in Plasma Lipids of Nephrotic Patients, J. Clin. Investigation 15:181 (March) 1936.

[7] Farr, L. E.: The Effect of Dietary Protein on the Urea Clearances of Children with Nephrosis, J. Clin. Investigation 15:703 (Nov.) 1936; The Assimilation of Protein by Young Children with the Nephrotic Syndrome, Am. J. M. Sc. 195:70 (Jan.) 1938; A Note on the Basal Metabolic Rate of Young Children with the Nephrotic Syndrome, Am. J. Dis. Child. 56:309 (Aug.) 1938.

[8] Howe, P. E.: The Determination of Proteins in the Blood: A Micro-Method, J. Biol. Chem. 49:109 (Nov.) 1921.

[9] Van Slyke, D. D.: Gasometric Micro-Kjeldahl Determination of Nitrogen, J. Biol. Chem. 71:235 (Jan.) 1927.

When the plasma albumin was below the edema level of about 1.2 Gm. per hundred cubic centimeters, restriction of salt caused no diuresis and had no marked effect in controlling the edema. In a small group of patients in whom the plasma albumin level ranged between 0.81 and 0.97 Gm. per hundred cubic centimeters, mercurial diuretics and urea used for a diuretic were not effective. In 1 patient urea was not effective when the plasma albumin level was 0.97 Gm., but became effective when the level rose to 1.47 Gm. per hundred cubic centimeters.



Summary of 114 observations on the plasma protein levels in 10 children with the nephrotic syndrome, showing relation of the plasma albumin level and the total plasma protein level to edema.

CONCLUSIONS

In children with the nephrotic syndrome there is a close relation between the presence of edema and the level of the plasma albumin in the blood. Edema can be controlled satisfactorily in most instances by simple restriction of salt, together with an adequate diet, when the plasma albumin level is above 1.2 Gm. per hundred cubic centimeters. This is decidedly below the critical value of 2.5 Gm. found for adults in this clinic.

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, March, 1939, Vol. 127, No. 3, pp. 617–625]

AN APPARATUS FOR THE RAPID AND ACCURATE DETERMINATION OF LOW OSMOTIC PRESSURES

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(Received for publication, November 28, 1938)

Some of the difficulties inherent in the determination of osmotic pressure by the methods already in use were felt most acutely in this laboratory when attempts were made to study human serum fractions both in normal and in nephrotic subjects.

In the technique used so successfully by Adair (1), a collodion bag containing the protein solution is tied to the lower end of a glass tube and immersed in a vessel containing the outer fluid. Several cc. of solution, a cold room, and much time are required (equilibrium being usually reached only after a week or two). The protein concentration must be measured in each bag after the end of the experiment, since some change in concentration always occurs. A determination of the specific gravity of the fluid and a correction for capillarity are also required. These complications render the accurate determination of low pressures rather hazardous.

For clinical purposes, many investigators, following Govaerts (2) and Krogh and Nakazawa (3), have used a different system, which involves a flat membrane (usually cellophane) and requires only a fraction of a cc. of solution. Two features of the method are that a counterpressure is applied on the inner fluid through a water manometer, and that the outer fluid is usually represented only by a moist disk of filter paper. We soon realized that this method, which is suitable for approximate clinical work, becomes useless if accuracy in the determination of low pressures is desired.

In more recent years, Oakley (4) has attempted to obviate some of the defects pointed out above. Unfortunately, his apparatus requires large amounts of material, and is very sensitive to temperature changes.

The method outlined below requires no more than 0.2 cc. of solution. At room temperature equilibrium is usually reached in 3 to 4 hours, and the final reading can be made from 3 to 4 hours later. There is no determination of concentration after the reading, no correction for specific gravity or capillarity. It is accurate enough so that a protein solution as little concentrated as 0.025 mM still gives readings which usually check within less than 5 per cent.

Description of Apparatus

The apparatus is essentially composed of the following parts:

1. A hard rubber part. This encloses a horizontal flat membrane separating the inner chamber (below), which contains the liquid to be examined, from the upper chamber (above).

2. A Y-shaped glass tube. The lower branch of the tube is connected with the inner chamber; one of the upper branches is connected through a stop-cock with the outer chamber; the other branch bears a capillary graduated in mm., which is the manometer.

The following description should be read with the help of Fig. 1.

The part containing the membrane is similar, if considered upside down, to that described by Krogh and Nakazawa (3). It is entirely made of hard rubber. The central piece *d* encloses the inner chamber *h* whose capacity is about 0.2 cc. and which is to receive the protein solution. The upper face of *h* is represented by the membrane *g*, followed by a perforated, hard rubber disk *f*, a thick soft rubber washer *e*, and cylinder *b*. All these parts are firmly clamped together by screwing *c* over *b* and *d*. This arrangement isolates tightly the inner chamber *h* from the outer chamber *h'*, permitting no protein solution to escape into the latter; at the same time crumpling of the membrane, which inevitably happens when the latter is in direct contact with a soft rubber washer, is here prevented.

At the bottom of *d* the arm *l* of the glass tube is inserted and firmly held in place by screwing the hard rubber cap *i*. The latter is separated from *d* by a hard rubber washer *k* and a soft rubber washer *j*.

The lower arm of the glass tubing is of 0.5 mm. inner diameter. It is bent twice at right angles, and before dividing into its two branches it widens into chamber *m* (0.05 to 0.1 cc. in capacity).

Chamber *m* is so placed that its floor, when the apparatus is assembled, is at the same level as the membrane.

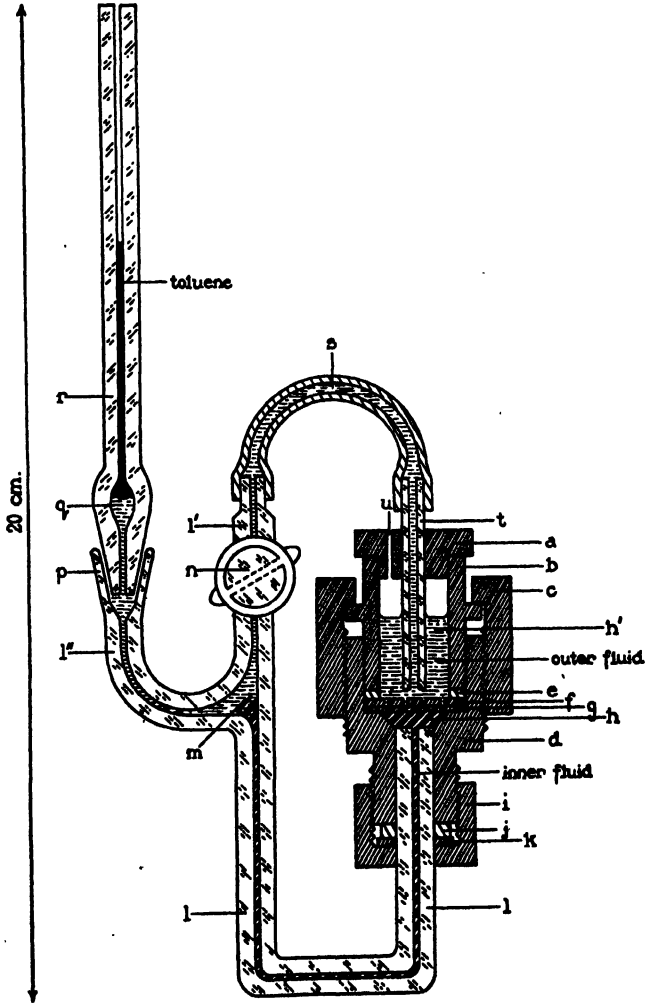

FIG. 1

One of the branches *l'* of the tube is provided with a 1-way stop-cock *n* which opens into a section of transparent rubber tubing, *s*, followed by a section of glass tubing, *t*, which passes through the hard rubber plug *a*. The latter bears a small hole *u* which insures atmospheric pressure inside chamber *h'*.

The other branch *l''* opens into cup *p*, which bears tube *r*. The latter is made of high grade 0.2 mm. capillary tubing, and is graduated in mm. The lower part of *r* widens into chamber *q*, the capacity of which is 0.05 to 0.1 cc. Capillary *r* is firmly plugged into cup *p* and contact takes place through carefully ground surfaces.

Description of Method

The apparatus is assembled and filled in the following way.

The glass tube is first fitted to *s*, *t*, *a*, and to *i*, *k*, and *j*, but not to *r*. The membrane, which has been dipping in the solution which is to be the outer fluid, is rapidly dried between two sheets of filter paper and immediately set in place in *d*, followed by *f*, *e*, and *b*. If the membrane is very thin, it is necessary to separate it from the perforated plate by a disk of filter paper, in order to give it perfect rigidity; the diameter of the paper disk should be slightly less than the upper diameter of the inner chamber. Then *c* is firmly screwed on. The object is now inverted and about 0.2 cc. of the solution to be tested is pipetted into *h*. Arm *l* is now introduced and pressed down until the fluid fills *l* as far as the entrance to chamber *m*. Cap *i* is then tightly screwed on.

The apparatus is now inverted again and placed in a support. Chamber *h'* receives about 1.5 cc. of outer fluid, *a* is plugged in place, and a slight negative pressure is applied over cup *p* so as to bring the fluid up through *t* and *s* and down through *l'*, *m*, and *l''* until it fills cup *p*. There should be no bubbles left in the capillary parts. A small bubble at the top of *s* is immaterial.

The manometer is prepared as follows: Its lower end is dipped in toluene, which is sucked up until it fills about the lower half of chamber *q*. It is then dipped in water, which is sucked up until the toluene fills the upper half of the chamber and the beginning of the capillary. The ground surface is lubricated with vaseline, and the capillary is plugged into *p*.

The whole procedure takes but a few minutes.

The apparatus should be placed in a water bath, the water just covering the top of *c*, and in a room at approximately constant temperature. It is now ready for operation.

The stop-cock being open, the toluene column in the manometer comes into hydrostatic equilibrium with the fluid in chamber h' through l'', l', s, and t. The pressure thus registered is p_1. As soon as the cock is closed the meniscus starts to rise, because to the hydrostatic pressure already present is now added the osmotic pressure which tends to transfer water through the membrane from h' to h, and therefrom to the manometer through l and l''. The rise of toluene will finally stop when the height of the toluene balances the sum of the hydrostatic and of the osmotic pressures. The figure then read is p_2. The osmotic pressure alone, in mm. of toluene, is $p_2 - p_1$. When, after the reading of p_2, the cock is opened again, the toluene falls back to p_1. Since some slight changes may have occurred in the whole system between the beginning and the end of the experiment, it is obviously preferable to take the second p_1 reading rather than the first.

TABLE I

Osmotic Pressure of a 0.1 mM Solution, Expressed in Mm. of Toluene, at Different Temperatures

Temperature	Pressure	Temperature	Pressure
°C.	mm.	°C.	mm.
15	28.0	23	29.0
16	28.2	24	29.2
17	28.3	25	29.3
18	28.4	26	29.4
19	28.5	27	29.6
20	28.7	28	29.7
21	28.8	29	29.8
22	28.9	30	30.0

Table I gives the osmotic pressure, expressed in mm. of toluene, developed by a 0.1 mM solution, in function of temperature. The calculations were based on the van't Hoff law and on the variation of the specific gravity of toluene with temperature (4).

The membrane being firmly held in place, no appreciable dilution can occur during the procedure. The amount of water which passes through the membrane until the final pressure is reached is exceedingly small. For example, a 0.1 mM solution will cause a rise of the toluene column of about 30 mm. The capillary being 0.2 mm. in inner diameter, the amount of liquid displaced will be $0.1^2 \times 3.14 \times 30 = 0.94$ c.mm. The capacity of chamber *h* being about 200 c.mm., this will mean a dilution of less than 0.5 per cent. A correction can be calculated for the purpose. For the same reason, the increase in pressure will not affect appreciably either the height of fluid in *h′*, or the capillary forces at the water-toluene interface in *q*.

Since the fluid to be tested fills entirely the U formed by the lower arm of the tube, and chambers *m* and *h* being at the same level, no correction for specific gravity is necessary. No capillarity correction is necessary for obvious reasons.

Toluene has been chosen because, besides being almost non-miscible with water, it is remarkably fluid and never adheres in the capillary tube (water for that purpose would be quite unsuitable). It should be noted that when the apparatus is dismantled the manometer should not be emptied, but kept as it is in a vertical position with its lower end dipping in water. It can thus be used indefinitely.

The apparatus can be conveniently used also for the determination of higher pressures by connecting, through a section of rubber tubing, the toluene manometer with a water manometer. The procedure then simply consists, after closing the stop-cock, in keeping the toluene meniscus approximately immobile by increasing progressively the counterpressure in the water manometer until equilibrium is almost reached. The apparatus is then left alone while the toluene meniscus climbs a few mm. and finally remains immobile. The final reading is given by the sum of the toluene pressure difference ($p_2 - p_1$) and the water counter-pressure applied. The specific gravity of toluene at room temperature is about 0.865 (5).

In order to test the apparatus, we prepared human hemoglobin solutions in the following way (6).

Oxalated blood was centrifuged, the serum removed, and the cells washed twice with 10 times their volume of 1.5 per cent NaCl. To 1 cc. of packed cells were added 0.3 cc. of water, 0.3 cc. of ether, and 0.2 gm. of solid NaCl. The solution obtained was centrifuged and filtered. It was then dialyzed against distilled water at room temperature in a little cellophane bag. The bag was held flat against a hard rubber frame, so as to offer the greatest possible surface in proportion to the volume of solution.

It was thus rocked in a trough for a few hours until the outer fluid, which was often renewed, remained salt-free. A mixture of Sörensen's phosphate solution was then added to the hemoglobin so as to make the final concentration M/30 and the pH 6.8. Under these conditions it is generally agreed (7) that the protein has a molecular weight of approximately 68,000. From this stock solution several more dilute solutions were prepared. They would keep in the ice box for weeks, without any apparent alteration. Spectroscopic examination showed no appreciable methemoglobin. As the proportion of total nitrogen, 16.75 per cent was used in the calculation (6). Total nitrogen determinations were made with the Van Slyke gasometric apparatus (8).

The difficulty of the method lies obviously in the use of a suitable membrane. Cellophane, of the kind which is quite satisfactory for dialysis, proved useless. With it, the pressure would rise a few mm., then stop abruptly and remain unchanged; no better result was ever obtained. With home-made collodion membranes the first results were not too unsatisfactory, but it was felt that a commercial product, if available, would give better guarantees of reproducibility. Therefore cellulose membranes manufactured according to Zsigmondy and listed as "ultrafine medium"[1] were tried and proved quite suitable.

Since these membranes are not very accurately graded, a few precautions are necessary. One large piece, as sold, can be cut into several small disks to fit the size of the apparatus. It is probably safe to test every one of these with a standard hemoglobin solution before using them further.

Since even membranes that seemed good at first sometimes later behaved abnormally, it was observed that plotting a time curve of the pressure change was helpful in eliminating erratic results, and that the two following conditions should be satisfied for the obtention of the most accurate figures.

1. The pressure-time curve should be quite smooth and regular, $\Delta p/\Delta t$ remaining always positive and decreasing slowly until equilibrium is reached.
2. Equilibrium should remain unchanged for at least 3 hours, with variations no greater than ±0.1 mm.

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[1] Sold by Pfaltz and Bauer, Inc., New York.

Generally speaking, when a result is wrong, the final figure cannot be read exactly; it usually comes out 20 or 30 per cent too low, and its time curve is flagrantly abnormal, so that it can be excluded most easily. Table II gives an example.

TABLE II

Molecular Weight Determination of Hemoglobin; Concentration 0.186 Per Cent; Temperature 21°

The values are given in mm.

Osmometer No.	1	2	3	4
11.25 a.m.	16.3	11.2	8.0	5.9
1.10 p.m.	22.9	16.2	13.2	12.0
2.20 "	23.2	17.3	14.3	12.6
3.27 "	22.9	17.9	14.8	12.9
4.40 "	22.4	18.1	15.0	13.0
5.55 "	22.0	18.2	15.1	13.0
6.40 "	21.9	18.2	15.1	13.0
9.00 "	22.1	18.1	15.0	13.0
(Stop-cocks opened)				
9.20 p.m.	16.3	10.6	7.7	5.4
$p = p_2 - p_1 =$	22.1 −16.3 5.8	18.2 −10.6 7.6	15.1 −7.7 7.4	13.0 −5.4 7.6
Mol. wt. $= \frac{23.8 \text{ mm.}}{p} \times 0.186 \times 10^5 =$		71,000	72,000	71,000

TABLE III

Molecular Weight of Human Hemoglobin in M/30 Sörensen's Phosphate Buffer at pH 6.8

Normal subject			Patient with nephrosis	
Concentration				
0.176 per cent	0.352 per cent	0.704 per cent	0.186 per cent	0.650 per cent
73,000	65,800	67,400	74,000	68,000
70,000	66,300	69,600	71,000	65,000
	70,000		73,000	67,100
	69,500		69,000	
	68,000		71,000	
			73,000	
			71,000	

Obviously the curve obtained from Osmometer 1 is unsatisfactory and should be rejected.

In the results given in Table III, all final figures read remained constant for at least 3 hours (sometimes much longer). As some of the determinations were allowed to stand overnight, complete time curves were not obtained for all, but there was no indication that important irregularities had occurred.

No particular claim is made as to the absolute accuracy of the molecular weights obtained, since they are reported primarily as an illustration of the method. The figures found for the smallest concentrations are by a few per cent above the molecular weight usually quoted. Whether this is due to a systematic experimental error (for which the membrane would most likely be responsible) or to the fact that at infinite dilution the correct figure is actually about 71,000 cannot be said yet. It should be borne in mind that the pressures read in that case are less than 8 mm. of toluene.

SUMMARY

An apparatus is described with which low osmotic pressures can be measured accurately on 0.2 cc. of solution and in less than 8 hours. Satisfactory results are obtained on solutions as little concentrated as 0.025 mM.

The method is illustrated with determinations of the molecular weight of human hemoglobin.

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[Reprinted from The American Heart Journal, March, 1939,
Vol. 17, No. 3, pp. 294–304]

THE EFFECT OF CERTAIN PURE DIGITALIS-LIKE GLUCOSIDES ON THE FROG'S HEART

By A. GARRARD MACLEOD, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research*)

(Received for publication, August 15, 1938)

In 1915, Cohn, Fraser, and Jamieson[1] described the characteristic effects of digitalis on the T-wave of the human electrocardiogram, and recommended the observation of these effects as a means of following the action of the drug in patients. The method has been applied extensively ever since in clinical medicine. It is surprising, therefore, that similar methods have been so little used in animals. Since the studies of such investigators as Straub,[2] practically no attention has been paid to changes in the form of the electrogram or electrocardiogram of animals under the influence of the drugs of the digitalis group. Lewis and his collaborators[3] and Love[4] used electrical records in investigating their effect on the refractory period of the heart, but did not study alterations in the form of the curves.

Since the researches of Dr. W. A. Jacobs and his collaborators[5, 5a] into the chemical constitution of certain digitalis-like glucosides have made available quantities of these substances in pure crystalline form, it seemed worth while to investigate their effect on cardiac action currents. Furthermore, the work of Craib,[6] Wilson, Macleod, and Barker[7] and Macleod[8] has made it possible to interpret electrograms rationally. A study of the effect of these pure glucosides on the frog's electrogram should give, therefore, an insight into their effect on certain of the fundamental properties of heart muscle, and consequently make it possible to understand more clearly the significance of the changes which they and similar substances produce in the human electrocardiogram.

Method

All of the experiments were performed on the Louisiana bull frog (*Rana catesbiana*). The animals were pithed, care being taken to avoid loss of blood. The heart was exposed by removing the sternum and slitting the pericardium, but left in situ. Electrograms were recorded from the surface of the heart by the unipolar method described by Wilson, Macleod, and Barker.[7] The exploring electrode (the one in contact with the heart) consisted of a thread, moistened with saline solution, projecting from the end of a silver tube whose inner surface was coated with silver chloride. The indifferent electrode (the one at a distance from the heart) was a silver plate, 1 cm. by 5 cm., coated with silver chloride, and it was placed beneath the skin of the hind leg. Since the resistance of the exploring electrode was high, a one-stage direct current amplifier was used in conjunction with the string galvanometer.

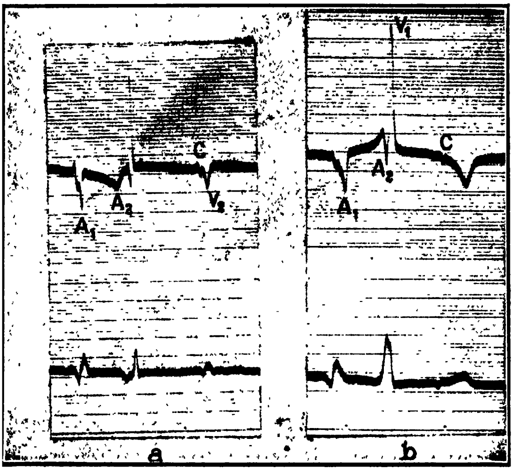

FIG. 1. The effect of atropine on the frog's electrocardiogram and electrogram. The upper curve in each case is a direct lead from the auriculoventricular junction. The lower curve is an indirect lead from the region of the larynx to the left hind leg. A_1, auricular accession deflection; A_2, auricular regression deflection (T-wave); V_1, ventricular accession deflection (QRS); V_2, ventricular regression deflection (T-wave); C, conal accession deflection. *a* was taken before the injection of atropine sulfate and *b* six minutes after 5 mg. were given into the lateral cutaneous vein.

During the experiment the thread projecting from the end of the silver tube was held in place by surface tension; it was sufficiently limber to follow the movements of the heart. In many experiments a cephalocaudal lead was also taken by placing an electrode in contact with the larynx and pairing it with the indifferent electrode.

When refractory periods were measured, a neon tube stimulator was adjusted to deliver shocks at a rate slightly slower than the existing heart rate. The stimuli were delivered to the tissue by means of a platinum electrode, the terminals of which were placed astride the exploring electrode. This method of obtaining the record from the region stimulated was adopted because Drury and Love[9] have

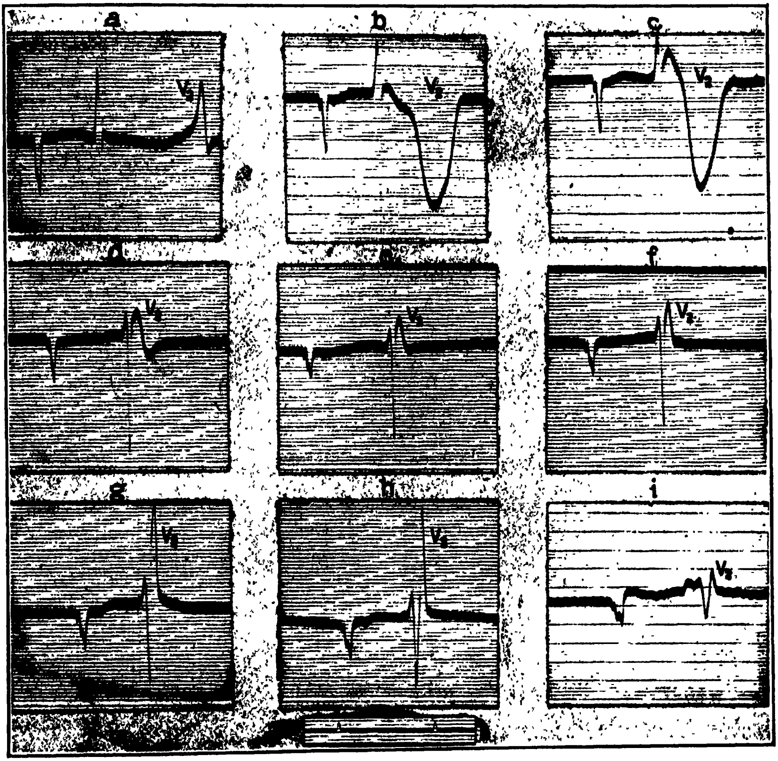

FIG. 2. The effect of cymarin on the ventricular electrogram. The exploring electrode was on the ventricle, almost at the A-V groove. V_2 is the ventricular regression deflection. *a* was taken before the administration of the drug. *b* was taken 12 minutes after a dose of 0.03 mg. of cymarin per 100 gm. of frog was injected into the lateral cutaneous vein. *c* was taken 20 minutes after the injection. *d* was taken 25 minutes after the first injection and 5 minutes after a second injection. *e*, *f*, *g*, *h*, and *i*, were taken 30, 34, 49, 56 and 66 minutes, respectively, after the first injection.

shown that an impulse arising just after the end of the refractory period may be conducted decrementally. A large deflection caused by the stimulus was recorded in the electrogram, but it did not interfere with the interpretation of the record nor endanger the string of the galvanometer.

The drugs were dissolved in alcohol and diluted to the proper strength with Howell's solution. The dose usually used was 0.03 mg. per 100 gm. of frog. This was sufficient to produce profound effects and was often lethal in from one and one-half to three hours. All drugs were given intravenously into the lateral cutaneous vein. The total volume injected in this way never exceeded 1 c.c.

It has been customary in studying the effect of digitalis bodies to eliminate the influence of these substances on the vagus mechanism by a previous administration of atropine. In most of the experiments here described this procedure was omitted. In the first place, it is generally agreed that in frogs the vagus nerves play no rôle in the action of these drugs and, secondly, it was found that atropine in doses of 1.0 mg. per 100 gm. of frog produced marked distortion of the electrogram (Fig. 1). This effect upon the complexes (a widening) is neither similar nor in antithesis to the change produced by the glucosides. Smaller doses did not influence the effect of the glucosides, although they abolished completely the effect of large doses of acetylcholine.

RESULTS

The first effect of cymarin on the ventricular electrogram (Fig. 2*b*) is inversion and increase in amplitude of the regression deflection (T) and shortening of electrical systole (Q-T). Later (Fig. 2*c*), an upward phase makes its appearance. Still later, the amplitude of the downward phase decreases rapidly until only the upward deflection remains. The duration of the regression process (T) steadily decreases, meanwhile, until (Fig. 2*h*) it is almost as brief as QRS. The primary change brought about by cymarin is, therefore, shortening of the duration of the T deflection, and consequently of electrical systole. As will be shown later, even the great inversion of T (Fig. 2*b*) can be explained as the result of shortening. Slight changes in amplitude occur in the QRS, but its duration is little affected.

To be certain that true shortening of electrical systole occurs under the influence of this drug, an area on the surface of the ventricle was burned after an unequivocal effect was produced with cymarin, and a monophasic response recorded (Fig. 3*b*). The duration of electrical systole is essentially the same in the monophasic curve as in the response obtained before injury (Fig. 3*a*). Both are short.

In order to ascertain if a similar effect is produced in auricular muscle, the exploring electrode was placed on the central part of the auricle. Since ventricular systole occurs before the auricular electrogram is complete, heart block was produced by Stannius' ligature.

When auriculoventricular dissociation has been established, the ligature may be released so that the force of the auricular contractions and the occasional ventricular beats will cause the circulation to continue even though it is greatly slowed. The effect of the drug in this case is similar, though perhaps less striking to that produced on the ventricle (Fig. 4). In this case the drug used was K-strophanthin-beta. Cymarin acts similarly. The illustrations have been picked purposely from experiments with different drugs in order to emphasize their close similarity.

It has long been thought that there is an association between the T deflection and the end of the refractory period (Mines,[10] De Boer,[11] Wilson and Hermann[12]). It seemed important to learn if this relationship persists when hearts are under the influence of these cardiac glucosides. The data in Table I indicate that it does. The first item is the average of a number of measurements of the duration of electrical systole and of the refractory period in a normal ventricle. The remainder of the table gives the results obtained in a single experiment in which the durations of electrical systole and of the refractory period were ascertained from time to time after the administration of cymarin. The shortening of electrical systole by the drug is accompanied by a

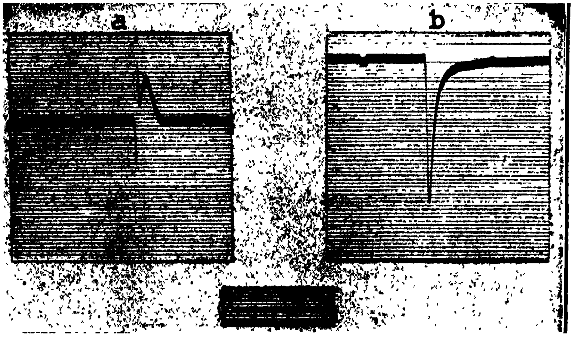

FIG. 3. The effect of cymarin on the monophasic response. *a* is an electrogram recorded from the central part of the ventricle 80 minutes after the injection of 0.03 mg. of cymarin per 100 gm. of frog. The duration of electrical systole is greatly shortened. *b* is an electrogram taken from the same spot as *a* after burning the area with a hot wire. The curve was taken about 10 minutes after *a*.

corresponding shortening of the refractory period (Table I). In this experiment the shortening of the refractory period was much greater than that observed by Love[4] in the ventricle of a tortoise under the influence of strophanthin, but the greater effect depends probably on the use of a proportionately larger dose. In the case of both electrical systole and the refractory period, shortening is followed by slight lengthening and, in turn, by shortening. This cyclic variation was very often encountered and frequently continued over quite long periods of time. Similar experiments on the auricle are more difficult to carry out, but the results are similar to those just described for the ventricle (Table II). In general, it may be said that the effect of the strophanthin glucosides on both auricles and ventricles is to shorten electrical systole and the refractory period.

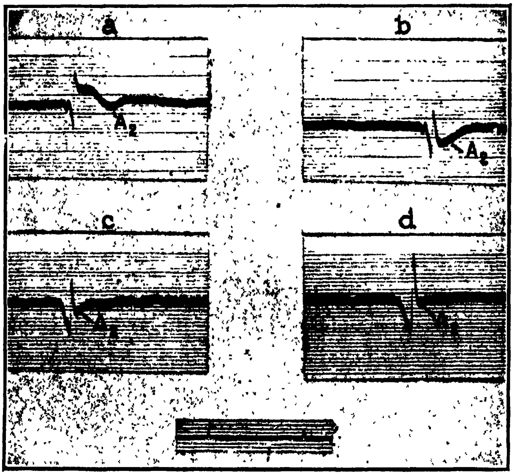

Fig. 4. The effect of K-strophanthin-beta upon the auricular electrogram. The exploring electrode was at the center of the anterior surface of the auricles. Heart block was produced fifteen minutes after the drug was injected. A_2 is the auricular regression deflection. *a* is the first curve taken after heart block was produced, about 18 minutes after the injection of the drug. Some shortening of electrical systole has already taken place. *b*, *c*, *d*, were taken 25, 35, and 45 minutes, respectively, after the injection.

Except for quantitative differences, the effects of the glucosides assayed (cymarin, K-strophanthin-beta, digitoxin, periplocymarin, and ouabain) were indistinguishable from each other.

In the later stages of their action, heart block was observed. The development first of ventriculoconal block and later of auriculoventricular block following the administration of periplocymarin is shown in Fig. 5. Sinoauricular block can also be observed late in an experiment after a large dose, but records showing this phenomenon are difficult to obtain because of the small amplitude of the sinus electrogram. Cushny[13] noted the development of sinoauricular block following the development of auriculoventricular block, but ventriculoconal block

TABLE I

		Duration of electrical systole (seconds)	Duration of refractory period (seconds)
Normal ventricle		1.15	1.04
Before cymarin		1.01	0.98
After cymarin	1*	0.69	0.48
	2	0.54	
	3	0.37	0.21
	4	0.47	0.34
	5	0.53	0.42
	6	0.34	0.28
	7	0.24	
	8	0.45	0.35
	9	0.41	0.21
	10	0.40	0.22

* Determination 1 was made about fifteen minutes after the administration of cymarin. The interval between the other determinations was about ten minutes.

TABLE II

		Duration of electrical systole (seconds)	Duration of refractory period (seconds)
Auricle before cymarin	1	0.77	0.60
	2	0.78	0.61
	3	0.80	0.63
Auricle after cymarin	1	0.24	0.21
	2	0.27	0.23

seems not to have been reported previously. This orderly development of block, first between conus and ventricle, then between ventricle and auricles, and finally between auricles and sinus, is of interest because it may be another example of the greater vulnerability of structures which develop later phylogenetically.

Occasionally rhythms suggesting auricular flutter or fibrillation occurred, especially when K-strophanthin-beta was given. These occurred at a time when the refractory period was short. Furthermore, in the process of studying the refractory period, multiple responses were often obtained to single early stimuli when the refractory period had been shortened by the administration of one of the drugs. This phenomenon was not observed in the normal heart.

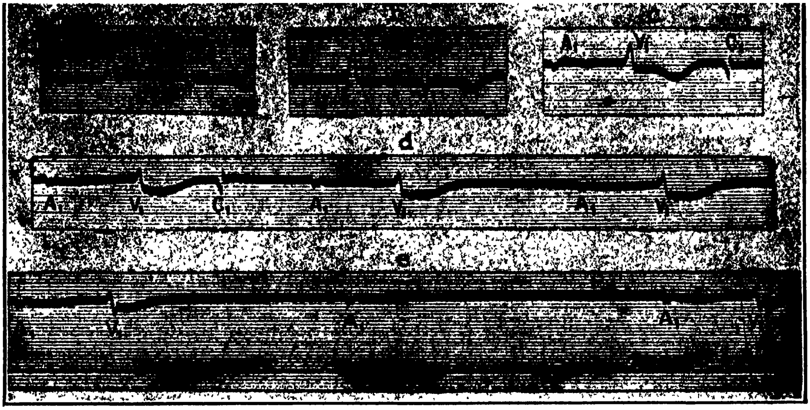

FIG. 5. The effect of periplocymarin on conduction. The lead is from the larynx to the left hind leg. A_1, V_1, C_1, are respectively the auricular, ventricular and conal accession deflections. *a* was taken before the administration of the drug. *b* was taken 2 minutes after 0.03 of periplocymarin per 100 gm. of frog. A-V and V-C intervals are slightly shortened. *c* was taken 10 minutes after injection. A-V and V-C intervals are lengthened. *d* was taken 50 minutes after injection; partial V-C block is present. *e* was taken 73 minutes after injection; both A-V and V-C block are present.

DISCUSSION

In 1913, Clark and Mines,[14] in a preliminary communication, reported that they had found a reduction in the duration of electrical systole and of the refractory period of the perfused frog's ventricle on the administration of strophanthin (probably on account of Mines' untimely death a fuller account of these observations was not published). Nevertheless, since then little importance has been attached to the duration of electrical systole. Recently, however, studies have appeared which indicate that in certain clinical conditions it is appreciably altered. Prolongation of the interval has been observed by White and Mudd[15] and Barker, Johnston, and Wilson[16] in conditions in which the calcium content of the blood is low. The opposite effect has been noticed by Cheer and Dieuaide,[17] who have demonstrated, by taking careful account of the variations in heart rate, a small but definite decrease in the Q-T interval following the administration of therapeutic doses of digitalis. Their results have recently been confirmed by Larsen, Neukirch, and Nielsen.[18] Observations on the refractory period of cardiac muscle under the influence of drugs are also not very numerous. It was originally held by Lewis and his collaborators that the refractory period of cardiac muscle was prolonged by strophanthin. Their observations were made on dogs whose vagi had been inactivated by atropine. Later, Drury and Love[9] and Love[4] showed that in hearts under the influence of veratrine, quinidine, or strophanthin, a stimulus falling just after the end of the absolutely refractory period gives rise to a response which may be conducted decrementally and consequently may not be detectable at a distance from its point of origin. Consequently, refractory periods ascertained by the use of older methods which judged the end of the refractory state by the first response that spread widely, rather than the first produced, may have been too long. Love[4] found that strophanthin actually shortened the refractory period of the ventricle of the tortoise. Lewis and Drury[19] have admitted the validity of this criticism of their earlier work, but maintain that what they had originally obtained was the "effective refractory period," and that this, rather than the true refractory period, was germane to their observations on the effect of quinidine and strophanthin on circus movement.

From the data of the experiments reported here it is impossible to ascertain whether conduction with a decrement occurred. A single direct lead was usually taken in the manner described. There was always a slight deformity of the electrograms caused by the stimulus when it fell at any time during the inscription of the curve. When a response was elicited a very definite modification of this artifact occurred, and a wave easily identified as T was apparent. T-waves under these circumstances furnish an unequivocal criterion of a response. Usually, when the heart was under the influence of the drugs an early response was followed, furthermore, by a paroxysm of fibrillation which could not, of course, fail to be detectable even at a distance from the point stimulated. Whether the early responses not accompanied by fibrillation would have been detectable in an indirect lead cannot be stated with certainty, for such a lead was not taken. These responses were large in the direct lead, but deflections in such a lead are, of course, largely produced by muscle very close to the exploring electrode. It is also possible, since early responses are smaller than later ones, that very early responses are so small that they are concealed by the stimulus artifact. While it seems probable that the changes in the refractory period here observed were gross enough to have been detected by any method, the question whether the early responses were conducted decrementally cannot be determined definitely from these experiments.

In a general way, the observations now recorded are in harmony with reports in the existing literature, although a true estimation of the value of these various results in establishing a generally accepted conception is difficult because the experiments have been performed on such a wide variety of subjects, from frogs to man.

Larsen, Neukirch, and Nielsen[18] refer to the experiments of Wiggers and Stimson,[20] in which it was shown that digitalis and strophanthin shorten the isometric contraction and ejection phases when these are ascertained from curves of intraventricular and aortic pressures, as corroborating their own observation that these drugs shorten electrical systole. It is inadvisable to place too much stress upon this point, however, for knowledge of the relationship between mechanical and electrical responses of heart muscle is incomplete. While a close relationship undoubtedly exists between the beginning of the two responses, their ends have not been shown to coincide. It is perhaps more correct to surmise that Wiggers' results, taken in conjunction with the observations that electrical systole shortens under the influence of drugs of the digitalis group, indicate that mechanical and electrical systole under these circumstances vary in the same direction.

This is an important relationship and more direct proof of its validity is highly desirable. That mechanical and electrical systole do not always vary in the same way is indicated by the finding of Barker, Johnston, and Wilson[16] that the marked increase in duration of electrical systole resulting from hypocalcemia is not accompanied by an increase in length of mechanical systole. Of the three entities, mechanical systole, electrical systole, and refractory period, the third appears to be closely linked to the second. The first may also be related to the second, but further evidence is necessary to establish to what extent and under what circumstances this correspondence can be relied upon.

It is the effect of these drugs on mechanical systole that is important to learn in order to decide whether benefit results from their administration apart from slowing the heart rate. The alteration of the refractory period is probably responsible for their well-known tendency to occasion auricular fibrillation and, in toxic doses, ventricular ectopic rhythms. It may also play a rôle in slowing the ventricular rate in auricular fibrillation. Changes in duration of electrical systole are important principally in so far as they may be used to measure alterations in one or the other of the properties just mentioned (mechanical systole or refractory period). They also may serve as a useful indication that the drug has had an effect.

Further, can the shortening of electrical systole which these drugs produce account for the changes in form (reduction in height or inversion of the T-wave) that have been observed by Cohn, Fraser, and Jamieson[1] and subsequent observers? It is possible that it can. The duration of activity differs, as is already known, in different parts of the heart. If it is shortened, it is not likely to be decreased precisely to the same extent in every region; for instance, it might be shortened most in muscle fibers in which it was initially longest. If this occurred, the order in which various regions pass out of the active state, and consequently the form of the T-wave, would be changed. The following explanation, which accounts for the change in form of the T-wave between Fig. 2*a* and Fig. 2*b*, will make this point clearer.

Before the administration of the drug, Fig. 6*A* may represent schematically the state of affairs. The muscle at the base of the ventricle upon which the electrode rests is the last to pass out of the active state, and the boundary between active and resting muscle is retreating toward *P*. Consequently, *P* is negative, but the boundary eventually passes under *P*, and now, since the positive side of the boundary is nearer *P*, this in turn becomes positive. The state of positivity is brief, for *P* is near the A-V boundary. If, now, a drug is given which shortens the duration of electrical systole and affects the muscle at the base ahead of the muscle elsewhere (possibly because it is thinner), the muscle at the base will pass out of the active state first, and Fig. 6*B* will represent the situation. The boundary will be retreating from *P* and consequently render it positive throughout the period of regression. This explanation is intended to show only that shortening of electrical systole can change the form of the T-wave. Further investigation is necessary before it can be stated that the changes observed in electrocardiograms of patients treated with digitalis actually are produced in this way.

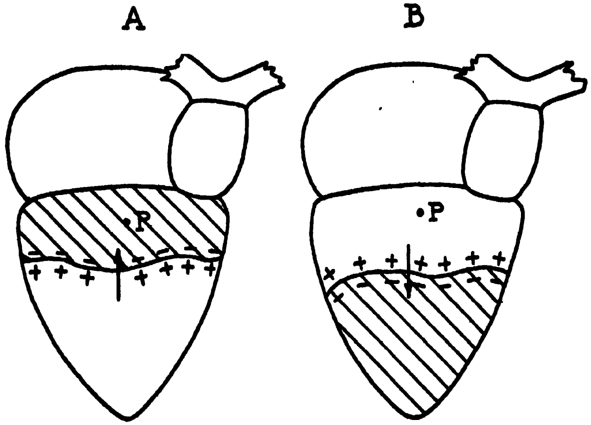

Fig. 6. Diagram of frog's heart to show change in course of the regression process resulting from local shortening in the duration of electrical systole. *P* is the point on which the electrode rests. The shaded region is the active muscle.

Finally, the close association between the duration of electrical systole and the length of the refractory period confirms the concept of Macleod[8] that the T-wave is produced by the recovery process. If this is true, the chief effect of these drugs is to hasten recovery. Recovery may not be so complete, however, as in the normal heart, for the QRS deflection is usually reduced in amplitude. This may be caused by a reduction in the potential difference between active and resting muscle, which in turn may indicate a reduction in the difference in their chemical and physical constitution.

SUMMARY

Certain glucosides of the digitalis group have been found to reduce the duration of electrical systole (Q-T) of both the auricles and ventricle of the frog. The refractory period is shortened pari passu with this reduction of electrical systole. It was not possible to bring out any differences in the mode of action of the various glucosides tested by the methods used.

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THE EFFECT OF ACETYL-BETA-METHYLCHOLINE ON THE FROG'S HEART

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The vagomimetic character of acetylcholine and closely related compounds has been demonstrated many times on many structures. In the heart the effect of vagus stimulation is quite complex. Gaskell[1] pointed out four aspects of the effect to which Engelmann[2] has given names: first, a reduction of heart rate (chronotropic); second, a diminution in the strength of contraction (inotropic); third, a decrease in the speed of impulse conduction (dromotropic); fourth, a decrease in excitability to direct stimulation (bathmotropic). To these must be added two other effects, a diminution in the duration of the excited state as evidenced by a reduction in the duration of the electrical response, described by Mines,[3] and the production of aberrant auricular rhythms, described by Lewis[4] and Robinson.[5]

In the study of acetylcholine and its relatives only chronotropic and inotropic effects have received much attention. The dromotropic effect has often been observed but has not proved a useful criterion of the action either of the vagi or of acetylcholine. The bathmotropic effect seems to be confined to the frog's heart and has not been much studied. Reduction in the duration of the electrical response and the production of ectopic rhythms, although highly characteristic of vagus stimulation, have scarcely been studied at all in connection with the action of acetylcholine and its relatives. It is important, however, to know if the cholines produce these effects, particularly if the drugs are to be given clinically. Goldenberg and Rothberger[6] have shown that large doses of acetylcholine produce something akin to auricular fibrillation in mammals. Abnormal rhythms may, therefore, occur in patients. While intense vagus stimulation is required to induce aberrant rhythms, Fredericq[7] has recently shown that a single shock to the right vagus nerve of the turtle will cause shortening of electrical systole in the auricle for several cardiac cycles. If the cholines also produce this change it is possible that the cardiac effect of the drugs can be observed and analyzed electrocardiographically.

Method

The experiments to be reported here were all performed on the Louisiana bullfrog (*Rana catesbiana*) at room temperature. The preparation and method of obtaining electrograms were similar to those described by Macleod[8] in his study of the strophanthin glucosides. The heart was exposed and a unipolar direct lead taken from either auricle or ventricle simultaneously with a cephalocaudal lead. The method used for measuring the refractory period has also been described.[8] The test stimulus was applied to the point from which the direct lead was derived.

The drugs were dissolved in Howell's solution and injected into the lateral cutaneous vein. Acetyl-beta-methylcholine (mecholyl) was used in a concentration of 0.01 mg. per cubic centimeter. As a rule, 0.1 c.c. of this solution was sufficient to bring on a marked effect in a large frog (300 to 400 gm.). The atropine solution used contained 1.0 mg. per cubic centimeter. One-tenth cubic centimeter of this solution would abolish the effect of acetyl-beta-methylcholine in theamounts used for a period of approximately three hours. By itself, this amount of atropine had no effect on the electrogram.

RESULTS

Following the injection of 0.1 c.c. of acetyl-beta-methylcholine solution the effect comes on quickly, often before the needle can be withdrawn. In some cases complete diastolic standstill occurs for a minute or more, in others merely marked bradycardia. With larger doses, periods of asystole of much greater length can be obtained. When an ectopic rhythm develops it usually occurs shortly after the period of most marked slowing. It is as a rule quite transient, but occasionally lasts for a considerable period of time (until the effect of the drug subsides). The maximum effect of the drug usually occurs within two minutes, and it passes off quite completely in about twenty minutes. Sinoauricular block often occurs but auriculoventricular block is seldom seen.

None of the electrograms showed dropped beats, but there was frequently moderate prolongation of the auriculoventricular and ventriculoconal intervals (Fig. 1*b*). In this respect acetyl-beta-methylcholine does not accurately imitate vagus stimulation, for stimulation strong enough to produce as marked sinus slowing as the drug produces

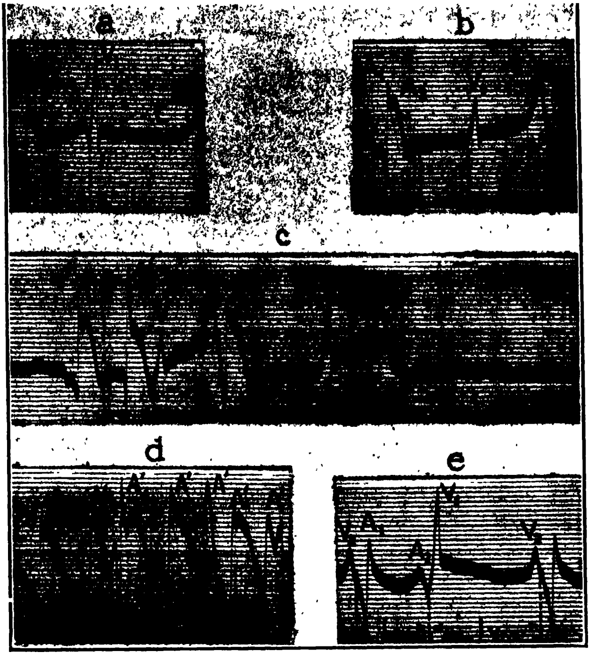

FIG. 1. Electrograms from frog's auricle. The exploring electrode was near the center of the anterior surface, but closer to the A-V than to the S-A junction.

a is the control before giving the drugs. A_1 is the auricular accession deflection. A_2 is the final part of the auricular regression deflection. Before it is quite complete, V_1, the ventricular accession deflection, occurs. V_2 is the final part of ventricular regression deflection. C is the conal accession deflection. The conal regression deflection is combined with V_2. l_A is the duration of the electrical response (electrical systole) in the auricle. l_v is the duration of electrical systole in the ventricle.

b is about one minute after giving 0.1 c.c. of acetyl-beta-methylcholine solution (0.01 mg./c.c.) intravenously.

c shows a spontaneous auricular extrasystole A′ during height of the drug's action.

d shows spontaneous auricular fibrillation, A_1 A_2 the last regular auricular complex. A′ S are aberrant complexes.

e shows the return to the original state after administration of 0.01 c.c. of atropine solution (1 c.c. = 10 mg.).

Curve	l_A sec.	l_V sec.	A-V interval	A-C interval
a	0.52	0.72	0.52	0.50
b	0.37	0.58	0.66	0.66
c	0.50	0.75	0.52	0.52

would certainly cause a much higher degree of auriculoventricular block.

The most striking effect of the drug on the electrogram is shortening of the duration of the electrical process in each of the chambers. Some change in shape of the accession* deflection is usually seen, but the deflection is not shortened. The shortening of the electrical response is brought about entirely by the decreased duration of the regression (V_2 or T) deflection.

The course of events in a typical experiment in which the exploring electrode was on the anterior surface of the auricle, near the center but somewhat closer to the auriculoventricular junction than to the sinoauricular, will illustrate these changes (Fig. 1). Auricular (Fig. 1*a*, A_1 and A_2), ventricular (V_1 and V_2), and conal (C) complexes can be made out. The drug was administered between the time of taking Fig. 1*a* and Fig. 1*b*, and the administration was followed immediately by a period of asystole. Because of this period it is impossible to follow the early changes in the regression process, for the drug has meanwhile had time to exert its full effect, so that when spontaneous contraction is resumed the change in form of the curve is already fully developed.

The auricular accession deflection (Fig. 1*b*, A_1) is somewhat changed in form and may be slightly increased in duration, but since its final portion is combined with the initial part of the regression deflection it cannot be accurately measured. This combination of the two deflections also contributes to the change in shape of the accession deflection. The regression deflection (A_2) is greatly reduced in duration and increased in amplitude. This change is similar to that observed by Macleod[10] under the influence of heat.

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* The frog's electrogram is the composite of the electrograms of each of its four chambers, sinus, auricle, ventricle, and conus, which beat seriatim. Of these only the sinus produces effects too small to be detected in the records. The electrogram of each chamber consists, furthermore, of two parts, a rapid deflection accompanying the accession of activity (the QRS of the human electrocardiogram) and a slower one accompanying regression of activity (T-wave). In a curve of such complexity the usual electrocardiographic method of naming the parts of the curve is entirely inadequate. In this paper, the terms accession and regression deflection will be used for the rapid and slow deflections, respectively, and will be modified by an adjective denoting their origin. This method of naming the parts of the curve was suggested by Macleod.[9]

In the case of the ventricle the accession deflection is decreased in size but is approximately of the same duration. But the terminal portion of the regression deflection (V_2) moves from a position to the right of the conus deflection (C) to a position between V_1 and C, and increases conspicuously in amplitude.

The conus complex is too small to be analyzed in these curves. Its regression deflection in this case cannot be made out. Under favorable conditions the complete complex can be seen, but the regression deflection is usually combined with the final part of the ventricular regression deflection or with the succeeding auricular complex. The effect of the drug on the conus seems, however, to be similar to its effect on the other chambers.

Auricular extrasystoles develop (Fig. 1*c*) and short periods of impure flutter or fibrillation (Fig. 1*d*). All of these phenomena are transient. The curve returns to its original form promptly on the administration of atropine (Fig. 1*e*).

It is sometimes possible to observe the changes in form of the curves as the effect of the drug progresses. In an experiment in which heart block had been produced by Stannius's ligature so that the *auricular complex* could be seen undistorted, the accession deflection (Fig. 2*b*), following the administration changed little except to decrease somewhat in size. The regression deflection at first becomes large and diphasic (Fig. 2*b*). Then, while retaining its diphasic character, it decreases in duration (Fig. 2*c*, *d*, *e*). In returning to its normal form as the effect of the drug wears off the curve does not follow a simple reverse course, but its final form is similar to the control.

In the case of the *ventricular complex* the principal change in the early stages of the action of the drug is as a rule marked inversion of the regression process with great increase in amplitude (Fig. 3*c*). The early ventricular effects are not so constant as in the auricle, probably because of the more complicated way in which the impulse spreads over this chamber. The regression deflection almost always increases in magnitude but is sometimes upright instead of inverted (Fig. 1).

The shortening of the duration of electrical systole suggests that the refractory period may be similarly shortened. Very accurate measurements are impossible when the effect of the drug is, as in this case, transient, and the duration of electrical systole is almost constantly changing. By repeated trials it was possible, however, to obtain satisfactory estimates. Two experiments, one on an auricle and one on a ventricle, are summarized in Fig. 4. The duration of

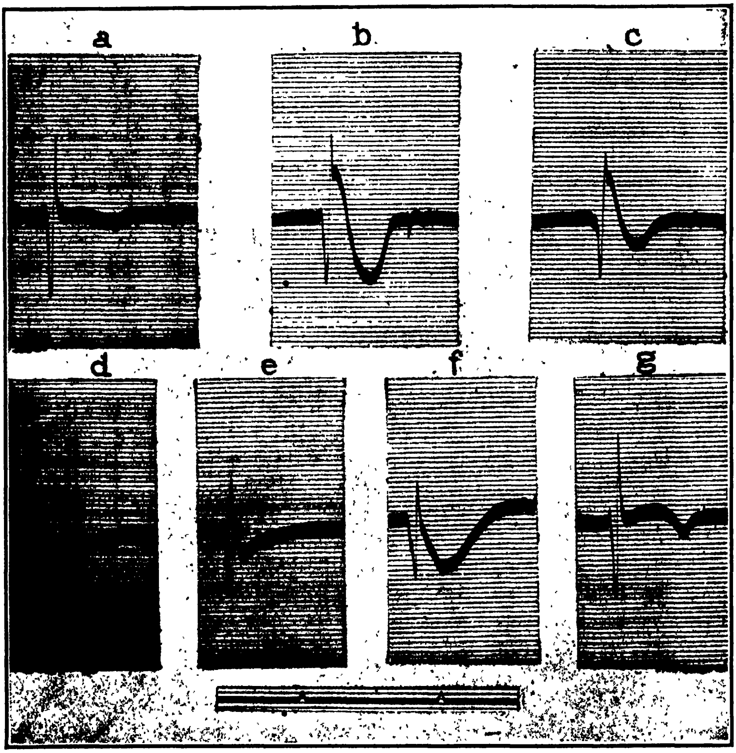

FIG. 2. Electrograms from the center of the anterior surface of the frog's auricle after auriculoventricular heart block was produced by Stannius's ligature. The auricular complexes are seen by themselves.

a, before administering any drug $l_A = 0.59$ second
b, 30 seconds after 0.1 c.c. of acetyl-beta-methylcholine solution (0.01 mg./c.c.) intravenously $l_A = 0.53$ second
c, 1½ minutes after injection intravenously $l_A = 0.47$ second
d, 2½ minutes after injection intravenously $l_A = 0.46$ second
e, 15 minutes after injection intravenously $l_A = 0.41$ second
f, 18 minutes after acetyl-beta-methylcholine and 30 seconds after 0.2 c.c. of atropine solution (1.0 mg./c.c.) $l_A = 0.64$ second
g, 5 minutes after atropine injection $l_A = 0.61$ second

the refractory period shortens pari passu with that of electrical systole, but is always somewhat shorter.

In the process of ascertaining the length of the refractory period an early stimulus often gave rise to multiple responses or to paroxysms of fibrillation or flutter (Fig. 5*a* and *b*). Such responses rarely occurred in normal hearts but were almost universal when hearts were under the influence of acetyl-beta-methylcholine. Aberrant rhythms fre-

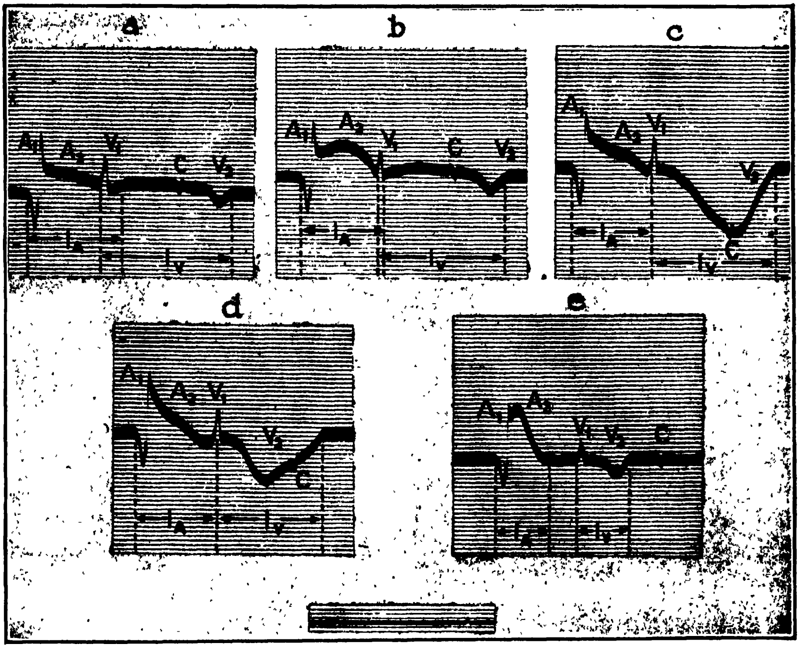

FIG. 3. Electrograms from center of anterior surface of frog's auricle similar to those in Fig. 1, but showing more typical changes in the ventricular regression deflection V_2, but very slight change in conduction time. 0.2 c.c. of acetyl-beta-methylcholine solution (0.01 mg./c.c.) was injected intravenously between *a* and *b*. The letters denote the same entities as in the previous figure.

Curve	l_A sec.	l_V sec.	A-V interval	A-C interval
a. control	0.69	1.00	0.53	0.56
b. 9 seconds after injection	0.66	1.00	0.58	0.56
c. 16 seconds after injection	0.63	0.95	0.58	0.58
d. 24 seconds after injection	0.61	0.81	0.61	0.58
e. 61 seconds after injection	0.40	0.40	0.61	0.60

quently occurred spontaneously in auricles (Fig. 1*d*) while they were under the influence of the drug, but were observed in ventricles only following an early stimulus (Fig. 5*b*).

A few experiments were performed with acetylcholine instead of acetyl-beta-methylcholine. The results were identical except that a larger dose was required to produce the same effect.

DISCUSSION

Mines[11] described experiments on the effect of muscarine on frogs' hearts. He found that the duration of electrical systole and the force of ventricular contraction were reduced, but that auriculoventricular conduction was little changed. Because of the close relationship between muscarine, acetylcholine, and acetyl-beta-methylcholine, Mines's results and those reported in this paper may be considered corroboratory. Mines noticed that muscarine did not occasion heart block. This observation has interest because this is the outstanding regard in which acetyl-beta-methylcholine fails to imitate vagus stimulation. As is well known, stimulating the vagi almost invariably produces prolongation of the auriculoventricular interval and, indeed, frequently causes partial or complete dissociation. But in none of the experiments of the present study was there ever more than a slight increase in the time of auriculoventricular conduction. The failure of these drugs (cholines) to be vagomimetic in this respect when given intravenously may depend on anatomic peculiarities of the frog's heart. Except the conus, no part of the frog's heart, as Grant and Regnier[12] have shown, is supplied with arteries; following injection into the blood stream, therefore, the drug reaches the myocardium by simple diffusion. Since the auriculoventricular bundle is embedded in tissue and is therefore removed from immediate contact with the blood stream, it is not directly reached by drugs. The sinus, on the other hand, is a thin structure not much covered over, so that it is very quickly acted on and exhibits the earliest effect of the drug in the form of sinus brachycardia or asystole. It is possible that the failure of these drugs to produce heart block is caused, therefore, not by their inability to affect the auriculoventricular tissue, but by their failure to reach it.

Heat, increase in rate of stimulation, and the drugs of the digitalis

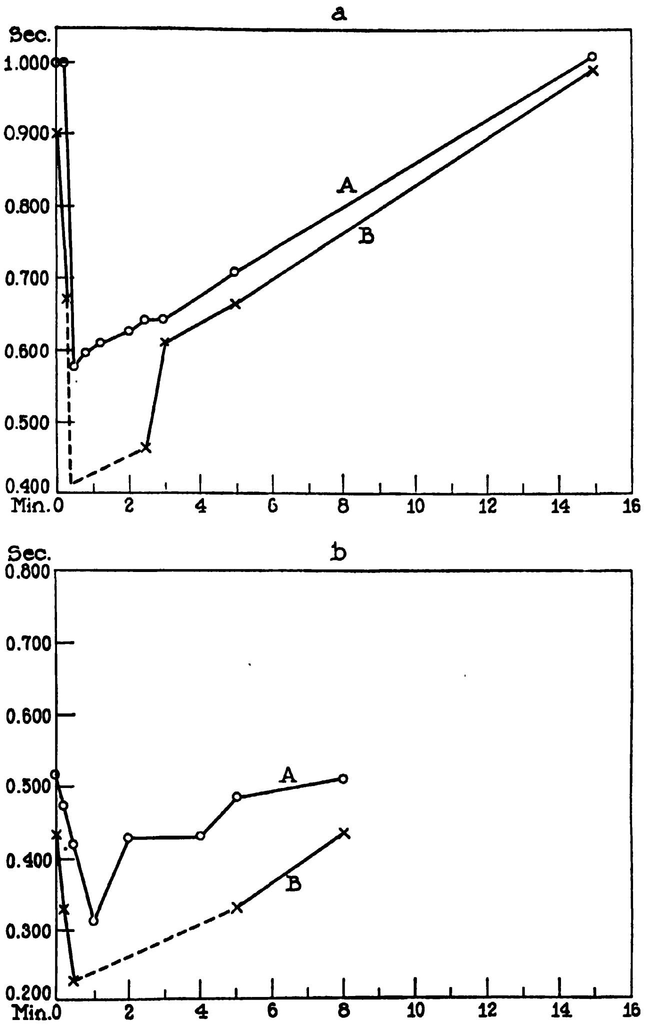

FIG. 4. *a*, Graph of the effect of injecting 0.1 c.c. of acetyl-beta-methylcholine solution (0.01 mg./c.c.) intravenously on the duration of the electrical response and the refractory period of the frog's ventricle. Curve A is the effect on the duration of the electrical response, and curve B the effect on the refractory period (the dotted portion of the curve is hypothetical).

Ordinates are the duration of the process in seconds.

Abscissas are the times after injection in minutes.

b, The effect of injecting 0.15 c.c. of acetyl-beta-methylcholine on the auricle. Curve A is the duration of the electrical response and curve B the length of the refractory period.

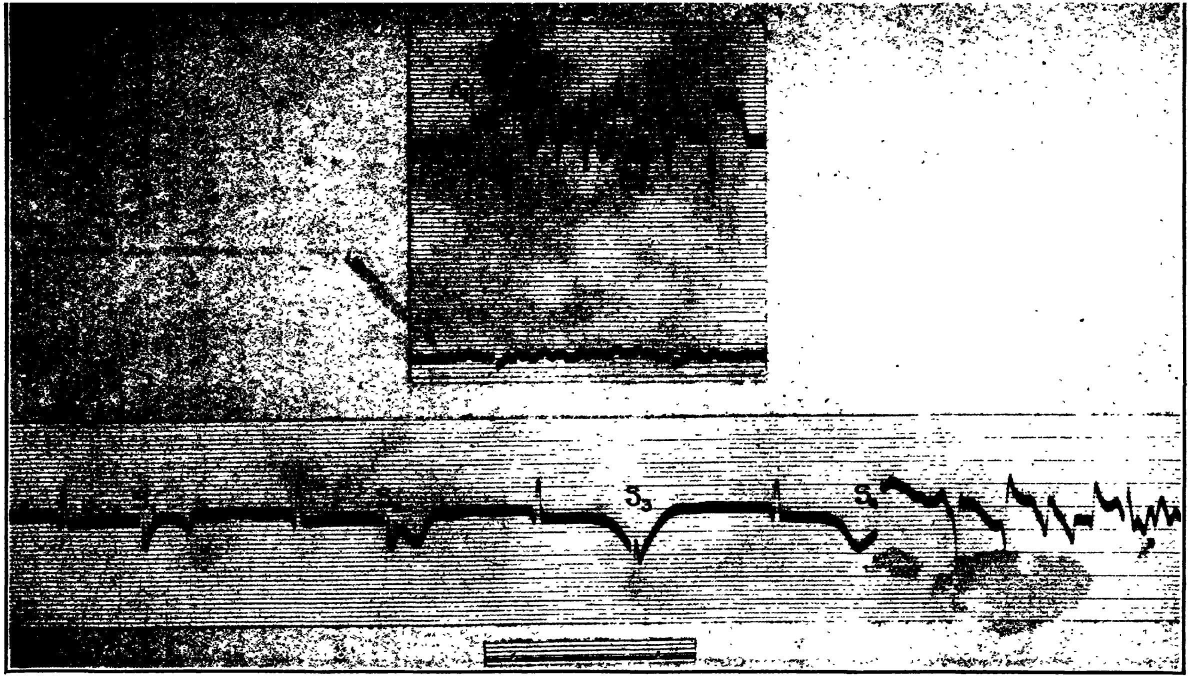

FIG. 5. Fibrillation produced by a stimulus falling shortly after the end of the absolute refractory period in a heart under the influence of acetyl-beta-methylcholine.

a, fibrillation of the auricle; S_1 and S_2 are stimuli.

b, fibrillation of the ventricle; S_1 S_2 S_3 are stimuli (this curve also shows the development of the effect on V_2).

group[8] are also capable of shortening electrical systole and in some instances of producing aberrant rhythms. Since so many physiologic functions have already been ascribed to acetylcholine, the question arises: Do any or all of these agencies act through the instrumentality of this substance? In the case of heat it is unlikely that shortening of the regression process is anything other than acceleration of the chemical reactions which bring about recovery. The progressive decrease in the duration of electrical systole and the refractory period with increase in the rate of stimulation is an interesting phenomenon for which no satisfactory explanation has been offered. The phenomenon in many ways resembles the effect of acetylcholine, but differs from it in that it is not abolished or opposed by atropine. The shortening caused by the digitalis drugs is also not antagonized by atropine. Acetylcholine or a closely related compound does not therefore seem to play a rôle in any of these phenomena.

In most of the experiments in this study the electrodes were arranged to record the events in both auricle and ventricle, and to bring out their time relationships rather than the form of the auricular electrogram. Curves such as those in Fig. 2 can, however, be compared fairly with those reported by Macleod[10] in his study of the form of the electrogram and its relation to the fundamental processes in cardiac muscle. The course of events in this experiment resembles that which he observed on the application of heat, but there are certain irregularities which require explanation. Particularly noticeable is the regression deflection of Fig. 2*f*, which may have been expected to resemble Fig. 2*b* but, instead, is almost monophasic. In attempting an explanation of this divergence from the expected form it is necessary to recall the conditions which must be fulfilled in order to obtain electrograms which correspond to Macleod's theoretic curves. In constructing these hypothetic curves only the existence of a narrow strip of muscle was assumed, with the excitation process travelling from one end towards the other. The curves obtained from a narrow strip resemble those from a wide one (the auricle) only if the duration of systole is the same for every muscle element and the speed with which the process travels is uniform throughout the muscle. If the duration of the excited state differs in different regions the curve may be greatly distorted. That the effect of the drug might persist much longer in some muscle elements than in others is easily possible, and would account for the failure of the curves to correspond to theoretic ones. It is in the transitional states during which the effect of the drug is increasing or decreasing that the most aberrant curves would occur. In a thin structure like the auricle, which is bathed uniformly with blood over one entire surface, absorption may be uniform, but the decay of the action depending on a number of factors would scarcely be the same for all regions.

The tendency of mecholyl to usher in fibrillation or flutter is the result of its effect upon the refractory period. Since Lewis[4] has discussed this relationship (the reduction of the refractory period and the occurrence of aberrant rhythms) at length, it need not be repeated here.

If the chief effect of acetyl-beta-methylcholine resembles that of the digitalis glucosides (a shortening of electrical systole), the two drugs may be expected to produce similar effects upon the human electrocardiogram. Page,[18] in his report on its clinical effects, illustrated the electrocardiographic changes. In the case of the subject whose curves are illustrated the electrocardiogram was normal before the drug was given but, during the height of the action, inverted T-waves, similar to those which digitalis produces, developed. The effect was, of course, transient, and the curve quickly returned to normal.

SUMMARY

Acetyl-beta-methylcholine shortens the duration of electrical systole and the refractory period in the frog's heart. In consequence, it may induce aberrant rhythms. These effects are abolished by atropine. Its failure to produce heart block is strange, but this can be explained on the basis of anatomic peculiarities of the frog's heart. It is probable that similar effects produced by frequent stimulation and the digitalis glucosides are not mediated by acetylcholine. Like the digitalis drugs, and probably by a similar mechanism, mecholyl causes inversion of the T-wave of the human electrocardiogram.

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PERIARTERITIS NODOSA

ITS CLINICAL RECOGNITION WITH REPORT OF AN ILLUSTRATIVE CASE

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Periarteritis nodosa was first described by Kussmaul and Maier (1) in 1866, but the condition remained a clinical curiosity for many decades. From then until 1937 about two hundred cases had been reported (2), and of these about twenty were diagnosed clinically (3). Many of these cases were recognized by means of biopsy study of a nodule.

Etiology. The cause of periarteritis nodosa remains obscure. The septic course of the disease suggests an infection, and its reported association with scarlet fever and tonsillitis (4) places the streptococcus in the foreground. Many observers maintain that the process is an allergic response (5) to a specific agent, such as the Streptococcus. The microscopic picture (6) of the disease lends further support to the theory of hyperergy. Gross and Friedberg (7) emphasize an interrelationship with rheumatic heart disease and Fahr's malignant nephrosclerosis. They believe that rheumatic fever is a common cause of vascular lesions which fit the description of periarteritis nodosa. Filterable viruses (8, 9), Rickettsias (10), and Spirochetes (11) have also been mentioned as causes. The etiologic factor, though still unknown, seems to be a specific agent capable of causing a typical vascular response. Whether this is an infectious agent or an allergen, only time and study will tell.

Pathology. This has been described as a necrotizing arteritis affecting chiefly the medium and smaller arteries. The earliest lesion is unknown, but is believed to be in the perivascular lymphatics of the adventitia (12). In gross specimens the process is recognized as a small yellow-red, smooth nodule (3) on the affected artery. Microscopically, it is characterized by an infiltration of plasma cells, lymphocytes, polymorphonuclear leukocytes, and eosinophiles. As the process advances, there is necrosis of the muscle cells in the media with the production of a fibrinous exudate and cellular infiltration of all layers by lymphocytes, plasma cells, and eosinophiles, and finally fibroblastic proliferation with invasion of small capillaries to form a scar. The amount of repair varies with the degree of the damage. Should the necrosis involve all the vascular layers with rupture of the elastic lamina, aneurysmal dilatation of the vessel wall develops which may progress to rupture of the aneurysm with hemorrhage into the surrounding tissues. This, in a rather high per cent of the cases, causes death, especially when it affects the medium-sized arteries. If the necrotizing process includes the intima, the latter responds with marked proliferation, thus paving the way for thrombus formation and varying degrees of occlusion of the vessel. Consequently, there is decreased blood supply to the involved tissue or organ resulting in ischaemia, infarction, or necrosis with subsequent atrophy. According to Gruber's (13) report of 108 cases in 1926, the organs affected in order of frequency were kidneys, heart, liver, gastrointestinal tract, skeletal muscles, pancreas, peripheral nerves, spleen, adrenals, gall bladder, and subcutaneous tissue.

Clinical picture. This is bizarre and protean in nature. Being a vascular lesion which may occur anywhere in the medium-sized and smaller arteries, it may simulate one or many other diseases at various stages of its development. An attempt to describe the clinical features precisely would be confusing and inaccurate. However, the symptoms may be divided into two groups.

1. Constitutional symptoms. A low grade fever, ranging up to 102 degrees is usually present and likewise a leukocytosis and a rapid pulse. Eosinophilia is occasionally encountered. Muscular pains are a predominating initial complaint, and with disuse of the muscles there are atrophic changes. Profuse sweats, marked weakness, loss of weight, and skin lesions are all prominent.

2. Local symptoms. Practically any organ may be affected. However, the symptoms are usually related to more than one viscus, and because of this the principal complaint of the patient may change from week to week. This is particularly important from a diagnostic standpoint. Should the process affect the kidney, we might have

symptoms and laboratory data to substantiate a diagnosis of Bright's disease. If this involvement is extensive and rapidly progressive it may simulate Fahr's malignant nephrosclerosis, unless symptoms referable to muscles, skin or other viscera place the diagnosis in doubt. Should the process involve the coronary or cystic arteries, signs and symptoms of coronary or gall bladder disease may ensue. When the patient is seen for the first time, the underlying pathology is seldom suspected. After the patient has been followed for several weeks, the bizarre picture and rapidly changing complaints, which are often related to the muscular system will make it clear that the process is a general rather than a local one.

Laboratory studies. These offer valuable aid in arriving at a diagnosis. As mentioned above, the low grade fever, leukocytosis, and eosinophilia are important clues. Secondary anemia may be present. Probably of greater significance than the anemia is the observation that a fall in the hemoglobin, red blood cell count, and blood pressure may follow an attack of sudden, severe pain in the abdomen. This was noted on at least two occasions by one of us in the case presented. It is probably the result of hemorrhage from a ruptured aneurysm. Since the kidneys are frequently affected, renal function tests are important. As the vascular damage is often extensive, the laboratory and clinical picture of Bright's disease is quite common.

Most intravitam diagnoses are made with the aid of a biopsy of muscle or a periarteritic nodule. This is undoubtedly the most important diagnostic aid. It is simple and inexpensive. Resort to biopsy should be had when any signs suggestive of periarteritis nodosa are encountered. It must be remembered that while a positive report is probably sufficient evidence for diagnosis, a negative one does not rule out the disease.

Prognosis. Considerable pessimism exists as to the future of patients with periarteritis nodosa. This may be because very few cases are diagnosed clinically, and if recognized are usually severe and far advanced. The average duration of life according to Gruber's report is 4.7 years. In all probability there are cases of undiagnosed periarteritis nodosa that go on to spontaneous cure.

Treatment. There is no specific therapy in this condition. General supportive measures and transfusions are of value. For relief of the muscular pains large doses of salicylates are effective. The arsphenamines are probably of value only if there is associated syphilis.

REPORT OF A CASE

Clinical Summary. The patient was a 34-year-old single male who had three admissions to the Brooklyn Hospital during the six months from January to June 1938. He was first admitted on January 4th, 1938 with the chief complaint of pain, spasm and stiffness of the leg muscles with associated loss of weight. He was discharged on January 22nd.

Present illness. The patient had had hypertension for the past sixteen years but never had any subjective symptoms. The systolic blood pressure varied between 180 and 240 mm. of mercury. Two years ago he first noticed mild attacks of pain affecting the muscles of his calves and thighs. These began as a "tensing of the muscles," then actual pain which progressed to a constant, sharp ache aggravated by any movement. The first attack lasted five days. During these two years he had five attacks which came at irregular intervals and became progressively worse both in severity and duration. Each attack was accompanied by fever. The longest acute episode lasted ten days. No predisposing cause was noted. During the entire illness he lost forty pounds in weight (200–160 pounds).

Past history. The patient had malaria 15 years ago but had had no symptoms from it during the past 14 years. He had diphtheria 18 years ago. There were no other serious illnesses, operations, or accidents.

His occupation was that of a civil engineer. He had traveled in Europe and South America.

Family history. His mother had died of cerebral accident. His father has hypertension. No familial diseases or allergic manifestations were known.

A review of systems was essentially negative.

Physical examination revealed a well developed, well nourished, adult male lying quietly in bed and not in apparent distress. The throat was moderately congested. There were a few soft, tender cervical lymph nodes. The heart was enlarged to the left and the sounds were of good quality. There was slight atrophy of the calf muscles, more marked on the left. These muscles were tender to touch. Active and passive motion was painful. The blood pressure ranged from 190 to 240 mm. Hg systolic pressure and 130 to 155 mm. Hg diastolic pressure. A low grade fever of 100 degrees was present.

Laboratory data. Chemical analysis of the blood showed: urea 43 mgm. per 100 cc. This was repeated three days later and found to be 25.0 mgm. per 100 cc. Creatinine was 2.2 mgm. Sugar 105 mgm. per 100 cc. Phenolsulphonephthalein and Concentration tests showed good kidney function. Blood count: Hemoglobin 93%, red blood cells 5,000,000; white blood cells 19,000 with 72% polymorphonuclear leukocytes, 25% lymphocytes, and 3% eosinophiles. The urine showed a faint trace of albumen on numerous occasions and also occasional white and red blood cells. The Wassermann test was negative. Biopsy of the gastrocnemius muscle for trichinae was negative and there was no evidence of arteritis. Teleoroentgenograms of the lungs, thighs and legs showed no pathologic conditions. There was one devitalized molar tooth. Tests with the thermocouple of the lower extremities were suggestive of arterial damage.

The course in the hospital was uneventful. The patient continued to have soreness and weakness in his legs. He was discharged planning to return after three weeks for tonsillectomy and removal of the devitalized tooth. During the interval at home a mass was discovered in the left groin. This was sausage shaped and measured 8 x 2 cm.

The second admission March 3 to May 19, 1938 was for tonsillectomy and because of the appearance of an evening rise in temperature to 101 degrees. Physical examination was essentially as above. The eyes were myopic. Both discs showed slight blurring of the nasal margins. In the right eye below the macula there was an area suggestive of old choroiditis with degeneration of the retina. Because of the fever tonsillectomy was deferred.

Laboratory data. The blood urea, creatinine, and sugar were normal. The red blood cell count dropped during the next few weeks from 5,000,000 with 94% hemoglobin to 3,660,000 with 67% hemoglobin. The leukocyte count ranged from 11,000 to 22,000 with a normal differential count. The eosinophiles reached 4% on one occasion. The urine continued to show albumin, white blood cells, and occasional red blood cells. Blood cultures were sterile. Repeated smears for malarial parasites were negative. The blood pressure ranged from 150 to 240 mm. Hg systolic and 130 to 155 mm. Hg diastolic. Roentgenograms of the pelvis and the right shoulder were normal.

Course in the hospital. During the first week the patient's main complaint was pain in the left groin at the site of the mass. An exploratory laparotomy was done, and the mass was found to be a retroperitoneal hematoma. The remainder of the abdomen was essentially normal. The patient continued to have bouts of weakness, air hunger, elevation of temperature to 101 degrees, profuse diaphoresis and migrating pains. At this stage one of us suggested the diagnosis of periarteritis nodosa and this was accepted as the explanation for the clinical picture. A prolonged fever in bouts over a period of two years accompanied by muscle pains had to be explained. When the retroperitoneal hematoma was discovered and shortly after this a severe pain developed in the region of the right kidney accompanied by the signs of hemorrhage, it became clear that we were dealing with an arterial disease with hemorrhage. Periarteritis nodosa explained all the symptoms and physical signs. During the next few weeks the patient complained of a multiplicity of symptoms ranging from swollen eyelids to clammy feet, but each day the predominating ailment was pain. This was referred to the shoulder one day, the leg the next day and included all parts of the body. Usually he complained of aching or soreness in the affected part, but on two occasions he had sudden, sharp pain in the right side, which made him catch his breath. With these attacks there was a substantial fall in the blood pressure and red blood count. These were believed to indicate hemorrhages in the right perirenal tissues and the gall bladder. There was no appreciable improvement during this hospitalization. The treatment consisted of sedatives, salicylates, calcium, quinine and neoarsphenamine.

The third admission was June 2, 1938, two weeks following discharge. He was readmitted because of right sided pain, nausea, vomiting, rising blood pressure, and the appearance of numerous red blood cells in the urine. The physical findings, laboratory data and treatment were essentially the same as on previous admissions.

The patient was transferred on June 6, 1938, his fourth hospital day, to the Hospital of The Rockefeller Institute for Medical Research for study of the hypertension. On June 7, 1938 the discovery of a pulsating mass and systolic murmur over it in the right upper quadrant of the abdomen led to a diagnosis of abdominal aneurysm. On June 8, 1938 at 3 A.M. the patient suddenly developed signs and symptoms of a severe abdominal hemorrhage and expired in fifteen minutes.

*Post-mortem examination:** The body was that of a well developed and well nourished white adult male.

Heart: The pericardial surface was smooth and glistening. The heart was enlarged (640 gms. in weight), due chiefly to enlargement of the left ventricle. Along the course of the descending coronary branches were numerous rounded sub-pericardial swellings, which on section were seen to be thin-walled, dilated sacs, filled with organized thrombi, and communicating with the lumina of the coronary arteries. They measured from 2 mm. to 6 mm. in diameter. Otherwise the epicardium was normal in appearance. The myocardium was firm, light red-brown in color, and contained many small hemorrhagic spots measuring from a fraction of a millimeter to 3 mm. in diameter. These areas had fibrous walls, were filled with organized thrombi, and some of them were seen to communicate with the lumina of vessels. They were very numerous and wide-spread. The coronary branches were in many places bulbous and tortuous. The endocardium and the valves were normal in appearance. There was considerable atheromatous deposit in the wall of the sinus of Valsalva, on the left interventricular septum, and in the ascending aorta and arch. There was a moderate amount of calcification in these deposits. Both main coronary arteries showed calcified and atheromatous deposits in the intima.

Lungs: The lungs showed no abnormalities.

Abdomen: The peritoneal cavity contained 1000 cc. of partly clotted blood. The sigmoid flexure and part of the descending colon were firmly attached to the parietal peritoneum by dense fibrous adhesions. Under the peritoneum the tissue was extensively infiltrated with clotted blood, probably measuring several thousand cubic centimeters in volume. The right lumbar gutter contained a retroperitoneal mass of fibrous tissue and old, partly organized, yellow thrombus, which distorted and bound down the retroperitoneal structures. There were extensive adhesions around the duodenum, gall-bladder, liver, and pancreas. In the region of the head of the pancreas was a mass of tissue closely bound to the duodenum. As the duodenum was dissected free a large cavity measuring 5 cm. in diameter, and containing partly clotted blood was opened. The wall of the cavity was thin and fibrous. Scattered over it, and throughout the head of the pancreas, were many irregular, raised nodules containing organized hemorrhages, and consisting of small cavities, the lumina of which communicated with arterial branches.

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* We are indebted to Dr. C. P. Rhoads for his kind permission to use his report of the post-mortem examination.

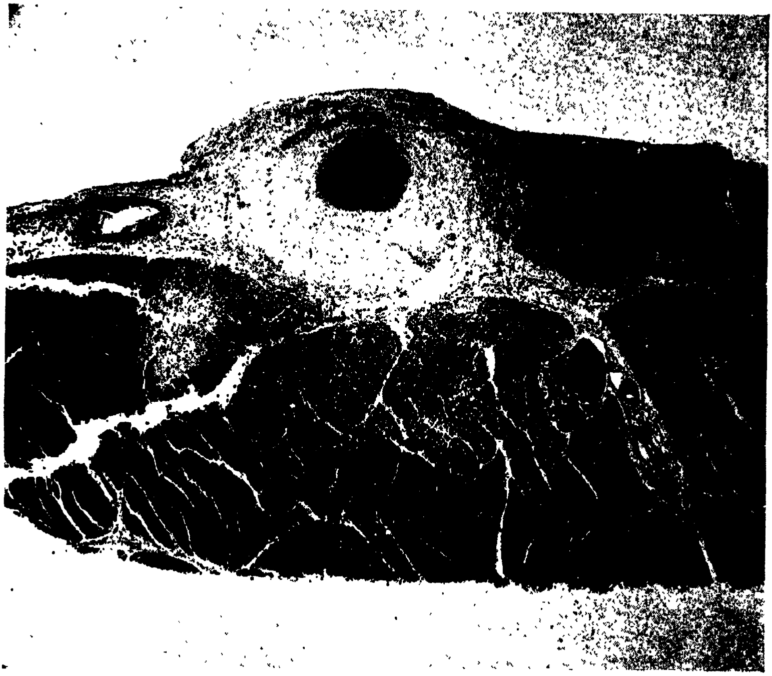

FIG. 1. Low-power view of a section of heart muscle showing a lesion in a thrombosed coronary artery

The large cavity itself was found to communicate with the site of the retroperitoneal extravasation, and with the pancreaticoduodenal artery, which in its course presented multiple small aneurysmal dilatations measuring 2 mm. to 12 mm. in diameter.

Similar small aneurysmal dilatations were found in the biceps muscle, along the brachial artery, in the gall bladder, and in the remainder of the pancreas.

Kidneys: Both kidneys were similar in appearance. The capsules stripped easily. There was considerable irregularity of the surfaces. The right weighed

190 gms. and the left, 185 gms. On section, the surface showed a striking abnormality. The grey-white background was flecked with tiny purple areas of hemorrhage. Many small dilatations of the branches of the renal arteries were found which were filled with organized thrombi. The main renal arteries showed an unusually large number of branches.

Spleen: The spleen was larger than normal, weighing 240 gms. The splenic artery was thickened and tortuous.

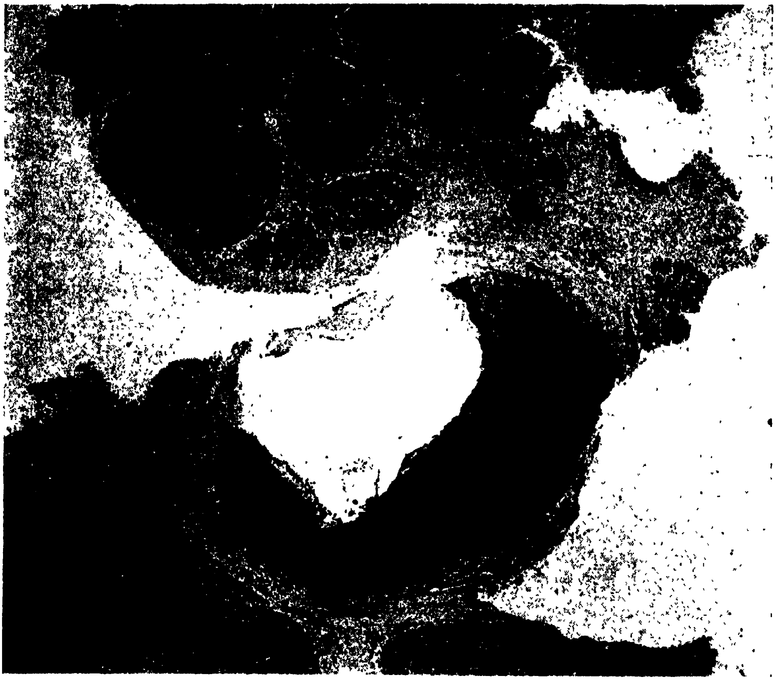

FIG. 2. Low-power view of a section of the pancreas showing a small aneurysm in a pancreatic artery

Liver: The liver weighed 2,280 gms. The surface was smooth. On section the surface was normal in appearance.

The adrenal glands, the abdominal lymph nodes, the gastro-intestinal tract, the bladder and genitalia, and the bone marrow, were not remarkable.

Microscopic examination: The *heart muscle* contained rounded areas of fibrous tissue surrounding branches of the coronary arteries, varying greatly in size. In these areas the intima had been practically replaced by fibrous tissue rich in cells. The media showed a striking degree of hyalin degeneration, in some instances so advanced that only occasional strands of muscle remained. The process of fibrosis involved the adventitia and extended beyond the location usually occupied by the adventitia. There was considerable infiltration of lymphocytes, with occasional polymorphonuclear leucocytes. In several places the fibrous tissue, rich in cells, had become infiltrated with red blood cells. Even the fine coronary branches showed hyalin degeneration of the media and replacement of the entire wall of the vessel with fibrous tissue. Apart from these periarterial lesions the heart muscle did not appear abnormal. The mitral valve showed a few small masses of fibrin on the surface of the endocardium.

Pancreas: In one section a cavity of considerable size, lined by very cellular, fibrous tissue, contained an organized thrombus. Other sections showed lesions of the pancreatic arteries similar to those in the coronary arteries. The media contained many hyalin deposits. Both media and adventitia consisted in many places of cellular fibrous tissue. The total thickness of the vessel wall in these lesions was greatly increased, and in some areas red blood cells could be seen breaking through the fibrous tissue. Many of the smaller branches of the pancreatic artery were thrombosed.

Kidney: There were many small but well-localized areas of scarring, occasioned apparently by ischemia. In several instances, branches of the renal artery in such areas were found thrombosed, the walls exhibited marked fibrous and hyalin thickening. Glomeruli in these regions were relatively free from blood, and the tubules were atrophied and crowded together. The interstitial tissue in these areas was decreased, and was thickly infiltrated with lymphocytes. Apart from the scars the renal tissue appeared well preserved, and the glomeruli and tubules appeared normal.

Abdominal lymph nodes: Sections of one node showed several areas of fibrous tissue replacing the normal structure, surrounding giant cells. The appearance suggested sclerosed tubercles. Sections of one other node showed nothing remarkable.

Sections of the lung, liver, spleen and bone marrow contained nothing remarkable.

Anatomical diagnoses: Periarteritis nodosa involving arteries in the heart, pancreas, kidney, gall bladder, skeletal muscle, and also the brachial artery. Aneurysm of pancreatico-duodenal artery with rupture. Aneurysm formation of coronary and pancreatic arteries. Retroperitoneal and intraperitoneal hemorrhage, old and fresh. Hypertrophy of heart. Fibrinous pericarditis. Healed tuberculosis of abdominal lymph node.

SUMMARY

A very unusual case of periarteritis nodosa diagnosed clinically, is reported. A biopsy specimen of muscle did not show any abnormalities. The disease involved the heart, pancreas, kidneys, gall bladder, skeletal muscles, brachial and pancreatico-duodenal arteries. The predominating initial complaint was muscle pain followed by weakness, loss of weight, diaphoresis, and attacks of sudden, severe abdominal pain. Leukocytosis and low grade fever were conspicuous features. Eosinophilia was not noted.

The diagnosis of abdominal aneurysm was made in the terminal stage. The necropsy findings are presented and confirm the clinical diagnosis.

CONCLUSIONS

A protracted disease with muscle pains, bouts of fever, sudden attacks of abdominal pain with abrupt drops in the red cell count should make one think of periarteritis nodosa. A biopsy specimen should be taken from one of the muscles that has recently been painful, but negative findings do not preclude a clinical diagnosis of periarteritis nodosa.

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MACROCYTIC ANEMIA IN ASSOCIATION WITH INTESTINAL STRICTURES AND ANASTOMOSES

REVIEW OF THE LITERATURE AND REPORT OF TWO NEW CASES

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The clinical picture of pernicious anemia in conjunction with intestinal strictures or anastomoses has been the subject of a number of reports since Faber first called attention to such an association in 1895. Reviews of the subject by Meulengracht in 1929 and Hurst in 1933 brought the number of recorded cases up to 30. We have collected 19 more cases from the literature to which we can add two of our own, thus raising the total number of cases to 51. The brilliant response of certain of the reported cases to surgical correction of the intestinal abnormality has furnished convincing evidence that the association between the intestinal lesion and the anemia is more than a coincidental one. However, considerable difference of opinion exists on two points: 1) the mechanism of the production of the anemia, and 2) the most effective form of therapy. It is our purpose to discuss these questions in the light of our own experience with two cases of macrocytic anemia occurring in patients with intestinal anastomoses and with the aid of information gathered in an analysis of the cases reported in the literature.

REPORT OF CASES

Case 49. D. S., male, aged 56, dentist.

History: The patient was admitted to the Hospital of The Rockefeller Institute on December 28th, 1935 with a complaint of weakness and diarrhea. He had enjoyed fairly good health until five years before. He had had the usual childhood diseases but no other serious infections except for right-sided pleurisy with effusion in 1917. He spent 6 weeks in bed at this time and did not work for 2 years. Tubercle bacilli were never found in his sputum.

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The patient's digestion was always rather easily upset and since youth he had suffered 8 to 10 attacks every year which were marked by abdominal pain, vomiting and diarrhea, lasting 2 to 3 days. Between such attacks he was subject to mild constipation. In the fall of 1930 his indigestion became worse, and he suffered with epigastric fullness, belching, and heart-burn after practically every meal. Various medicines were tried without effect. In February, 1931 a laparotomy was performed and a fecalith was removed from the ileum. The lower ileum was found to be thickened and indurated. The patient's blood count was normal at this time. He experienced some relief following the operation, but in the spring of 1932 the abdominal complaints again became severe and the constipation became more marked. In June 1932, 18 inches of the lower ileum were resected. The pathological diagnosis was non-specific ileitis. He recovered slowly from this second operation but was eventually able to return to work. In June 1934, 2 years later, he was forced to stop work because of weakness. A mild macrocytic anemia was noted at this time, which did not respond appreciably to weekly injections of liver extract or to iron by mouth. In November 1934 a diagnosis of partial intestinal obstruction was made, and the patient was operated on at the Mayo Clinic, where an anastomosis between the lower ileum and the transverse colon was performed. There was well marked macrocytic anemia before the operation, which failed to respond to parenteral liver extract. The red blood cell count rose rapidly after the operation and he was discharged with a mild hypochromic microcytic anemia. However, he soon developed diarrhea with three to six yellow foamy stools a day, which persisted up to the time of his admission to this hospital. There was marked flatulence and gaseous distention. The rectum was often quite sore, and occasionally he experienced burning of the tongue. He developed increasing weakness and pallor. There had been a loss of 40 pounds in weight over the 5 year period of his illness, although his diet had been excellent.

Physical Examination: The patient was a pale, emaciated, white man who was not acutely ill. Weight 45.7 kg. There was slight edema of the feet. The tongue and buccal mucosa appeared normal. There was evidence of thickened pleura and chronic bronchitis over the lower right lung. The heart and blood pressure were normal. The abdomen was distended and boggy. There were numerous operative scars and diastasis recti. Intestinal patterns were readily visible. There was no abdominal tenderness and no abnormal masses were felt, although there was some resistance and fullness in the right lower quadrant. Neurological examination was quite normal except for questionable slight diminution in vibratory sensation in the feet.

Laboratory Findings: Blood count showed—R. B. C. 2,350,000, hemoglobin 69%, W. B. C. 3,800, M. C. V. $124\mu^3$, C. I. 1.47, icteric index 3, reticulocytes 2%. Differential was normal except for 6% eosinophils. Platelets 520,000. The smear showed definite anisocytosis with a preponderance of hyperchromic macrocytes; little poikilocytosis. *Urine* was normal. The *stools* were liquid, gray-brown in color, with occult blood on occasions; excess fat was present. *Gastric analysis:* No free hydrochloric acid in the fasting specimen, but abundant free acid after alcohol test meal, and a rise to 87° after histamine. *Sternal marrow biopsy* revealed a red, highly active marrow (Figs. 3A and 4A). Supra vital differential count (500 cells) showed:

	per cent
Polymorphonuclears	28.7
Myelocytes (chiefly C)	15.1
Eosinophils	5.0
Normoblasts	18.0
Late Erythroblasts	14.7
Early Erythroblasts	9.5
Megaloblasts	2.5
Primitive Cells	6.5

Sections of the marrow showed marked erythro-megaloblastic hyperplasia such as is seen in the marrow of cases of pernicious anemia in relapse.

Liver Function: (1) Bilirubin excretion test: Patient given 50 mg. bilirubin intravenously. No retention after four hours. (2) 24 hour urine urobilinogen excretion 0.247 mg., a normal value. (3) Sodium benzoate test of Quick: 1.8 Gm. benzoic acid excreted in 4 hours, an abnormally low value suggesting liver damage or possibly poor absorption.

X-rays of gastro-intestinal tract revealed a normal stomach and showed the anastomosis between the ileum and transverse colon to be functioning well. The blind loop, consisting of the proximal part of the transverse colon, the ascending colon and the terminal ileum (Fig. 1), filled by retrograde flow of the barium. The terminal ileum was obviously diseased, with several large lakes of barium separated by narrow strictures.

Course: It was apparent that the patient was suffering from a sprue-like condition, with a well marked macrocytic anemia, and the question arose as to whether the deficient absorption of hematopoietic material or the absorption of toxic substances from the blind loop might be responsible for the anemia. A number of tests of intestinal absorption were carried out: 1) The potassium iodide test of Heath and Fullerton gave an appearance time of ten minutes for iodine in the saliva, a rapid normal value. 2) Glucose tolerance test showed a rise in blood sugar from 95 to 221 mg. per 100 cc. at the end of one hour with a slightly delayed fall.

The patient was given 1.25 Gm. of glucose per kilogram by mouth and the following figures for blood sugar were obtained:

Specimen	*Blood Sugar*
Fasting	95 mg. per 100 cc.
½ hour after glucose	211 mg. per 100 cc.
1 hour after glucose	221 mg. per 100 cc.
2 hours after glucose	139 mg. per 100 cc.
3 hours after glucose	139 mg. per 100 cc.

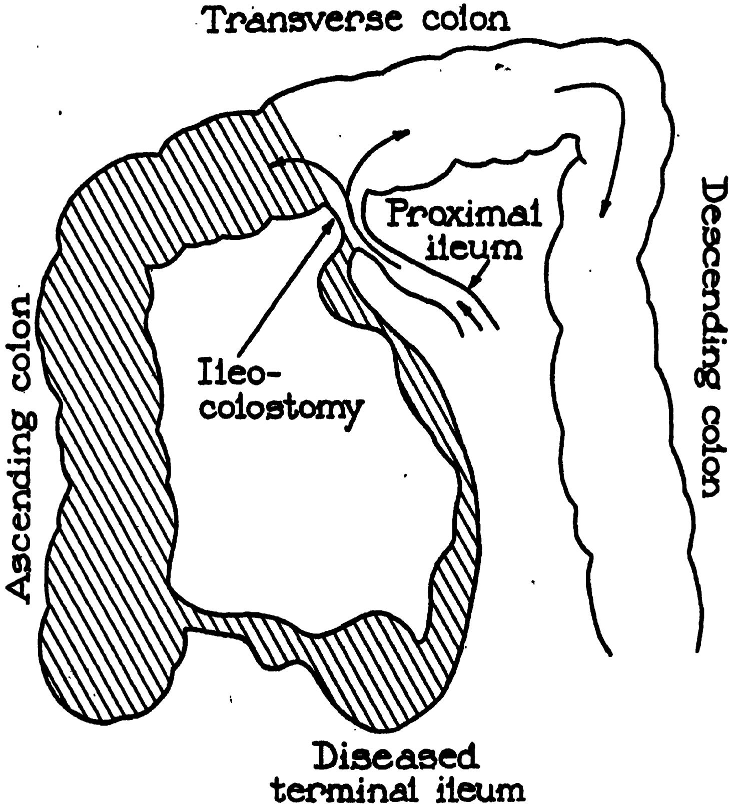

FIG. 1. *Case no. 49. Diagrammatic representation of the ileocolostomy.* The shaded area represents the short-circuited loop of bowel; the arrows show the course followed by the intestinal contents.

3) Fat absorption following a fat meal furnishing 4 grams of fat per kilogram of body weight showed 34% increase in the total plasma lipids after 4 hours. The figures obtained in the test were as follows:

Specimen	*Total plasma lipid carbon*
Fasting	403 mg. per 100 cc.
2 hours after fat meal	465 mg. per 100 cc.
4 hours after fat meal	539 mg. per 100 cc.
6 hours after fat meal	454 mg. per 100 cc.

This result compares favorably with the absorption of fat in normal individuals (Barker and Rhoads). The ingestion of the meal caused no unusual gastro-intestinal disturbances. 4) Ascorbic acid absorption: The patient was placed upon a diet low in vitamin C, and the 24 hour urinary excretion of ascorbic acid was followed. We are indebted to Dr. Julius Sendroy for carrying out the determinations on the urine. On the low vitamin C diet alone the patient excreted between 35 and 45 mg. of ascorbic acid in 24 hours. The diet was then supplemented with 250 mg. of crystalline ascorbic acid a day; there was no rise in the amount excreted over a 7-day period. Oral administration was then discontinued and 250 mg. of ascorbic acid were given intravenously each day for the next 7 days. Urinary excretion rose to 175 mg. on the 4th day and remained at a level between 160 and 250 mg. a day for the remaining 3 days of the test period. These results suggested deficient absorption of the ascorbic acid, destruction of the vitamin in the intestinal tract, or an unusual degree of unsaturation on the part of the patient. The patient was very uncomfortable while taking the vitamin C-free diet for study of his ascorbic acid metabolism. The erythrocyte count fell to 1,650,000 and the hemoglobin to 55% during this period, and his tongue became red and sore. The patient's reticulocytes were followed daily and on February 2nd he was given a diet for sprue, high in protein and low in carbohydrate and fat (Miller and Barker). Reticulocytes rose to 6.8% on the seventh day of this diet. Autolyzed yeast,[1] 15 Gm. a day, was then added and a second reticulocyte rise to 8.5% occurred. This reticulocyte rise during the administration of foods rich in Castle's extrinsic factor was regarded as evidence that the patient was secreting intrinsic factor in his stomach. Unfortunately it was impossible to test his gastric juice directly for intrinsic factor. The red blood cell count rose to 2,500,000 and the hemoglobin level to 70% on the regime of the diet for sprue and autolyzed yeast. When desiccated hog's stomach,[2] 20.0 Gm. a day, was added, the reticulocytes remained elevated between 4 and 8%, but there was no further rise in the erythrocyte count or hemoglobin. Finally the patient was given liver extract,[3] 12.0 Gm. a day by mouth (the amount derived from 300 Gm. of whole liver) and the reticulocytes once more showed a rise to 7.5%, after which they gradually dropped off to a level near 2%.

The patient was subjectively a good deal better while taking the liver extract by mouth, but mild diarrhea and flatulence persisted and the erythrocyte count did not rise above 3,500,000 with the hemoglobin level remaining at 70%. Therefore he was given liver extract parenterally, at first intravenously and later intramuscularly. There was a striking improvement in the gastro-intestinal symptoms and a further rise in the blood count during the period of parenteral liver extract administration. The course of the blood changes is depicted in figure 2. The patient was discharged from the hospital on May 8th, 1936. At the time he was feeling very well, with practically no diarrhea and very little flatulence. He had

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[1] The preparation used was Vegex.

[2] The preparation used was Ventriculin.

[3] The preparation used was Liver Extract, Lilly, N. N. R.

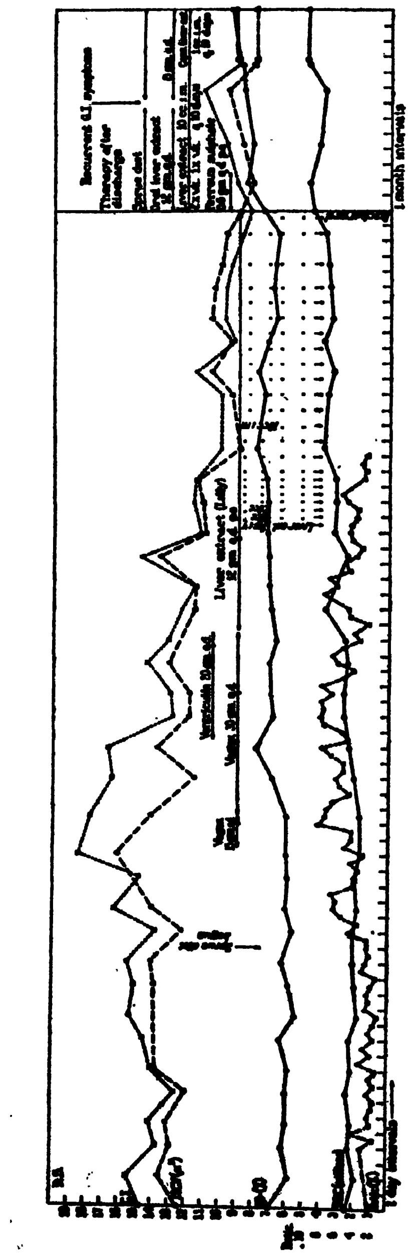

FIG. 2. *Case no. 49. Course of the blood under various forms of therapy.* On the scale, the values for Hb. (hemoglobin in percentage of normal) and M. C. V. (mean corpuscular volume) have been divided by 10, and the values for C. I. (color index) have been multiplied by 10.

gained 6.0 Kg. in weight during his stay in the hospital. At the time of discharge his red count was 4,550,000, hemoglobin 75%, and white count 7,900. Mean corpuscular volume and color index were both slightly lower than normal. For this reason small amounts of iron were added to his therapy.

He was sent home on the following regime:

(1) Sprue diet.
(2) Liver extract, three teaspoonfuls a day.
(3) Ferrous sulphate 0.2 Gm. three times a day.
(4) Intramuscular injection of 10.0 cc. liver extract, (the amount derived from 50.0 Gm. whole liver) twice a week.

Course following discharge from the hospital: Following discharge from the hospital the patient adhered closely to his therapeutic regime and continued to do very well. He was up and about the entire day. Gastro-intestinal symptoms did not recur. In September 1936 his blood count was as follows: R. B. C. 4,220,000, Hgb. 82%, W. B. C. 6,150, M. C. V. $88\mu^3$, C. I. 0.97. The interval between the liver extract injections was gradually lengthened to ten days. Toward the end of November he felt so well that he decided to abandon his diet and the oral liver extract, believing that the intramuscular injections would suffice to keep him symptom-free. Within a week of discontinuing the oral therapy, he developed abdominal cramps, diarrhea, and flatulence. These symptoms became so troublesome that he returned to the sprue diet and oral liver extract of his own accord. On December 21st, 1936, his blood picture once more showed a tendency to macrocytosis and hyperchromia, evidently associated with the flare-up of gastro-intestinal symptoms: R. B. C. 3,950,000, Hgb. 87%, W. B. C. 7,700, M. C. V. $95\mu^3$, C. I. 1.10. The gastro-intestinal symptoms cleared up within several weeks of this time. On February 15, 1937, he was readmitted to the hospital for further observation.

Physical Examination: The patient appeared vigorous and cheerful. Nutrition good, weight 52.8 Kg., no pallor or jaundice. Tongue normal. Bronchitic râles over the right lower lobe. Abdomen flat, soft, non-tender. Movable mass in the right lower quadrant suggesting a fecalith in the cecum or terminal ileum. Neurological examination normal.

Laboratory Findings: Blood count: R. B. C. 4,880,000, hemoglobin 88%, W. B. C. 7,000 with normal differential. M. C. V. $81\mu^3$, C. I. 0.90, icteric index 5, reticulocytes 0.4%. Smear: Red cells appear normal; platelets abundant. *Urine:* Negative. *Stool:* Semi-formed; no gross blood; guaiac +. *Barium Enema:* Appearance of colon and ileum identical with that of a year before. Ileo-colostomy patent and functioning well.

Liver Function: 1) Serum bilirubin 0.3 mg. per 100 cc. 2) Bilirubin liver function test: No retention after 4 hours. 3) Sodium benzoate liver function test: 3.453 Gm. benzoic acid excreted in 4 hours, a normal result in contrast to the very low output at the time of his first admission.

Sternal Marrow Biopsy: Specimen of marrow appeared quite cellular in the gross, with few spicules of bone. The supravital differential count (500 cells):

	per cent
PMN	50.0
Myelocytes B	0.2
Myelocytes C	18.8
Basophils	0.6
Eosinophils	4.8
Normoblasts	20.0
Late Erythroblasts	2.4
Early Erythroblasts	0.2
Primitive Cells	2.4

Sections of the marrow (Figs. 3B and 4B) presented an essentially normal picture. There was considerable fat present; no megaloblasts were seen in contrast to the striking megaloblastic hyperplasia noted at the time of the previous biopsy.

Course: The patient was discharged 5 days after admission to continue on the same therapeutic regime with the exception that a more concentrated liver extract preparation was to be substituted for the dilute extract previously used. In the spring of 1938, two years after his first admission, he again complained of mild gastro-intestinal symptoms. Since for economic reasons he had cut down the amount of liver extract he was taking, it seemed likely that this most recent recurrence of symptoms was due to inadequate therapy. When last heard from, he was once more symptom-free.

Summary: A 56 year old white man had suffered from symptoms of partial intestinal obstruction due to non-specific ileitis for 4 years. Shortly after an ileocolostomy was performed for relief of the obstruction, the patient developed a severe macrocytic anemia, associated with diarrhea, flatulence, glossitis, and loss of weight. The chief physical findings were pallor, emaciation and abdominal distention. There were no neurological changes. The blood picture was that of a severe macrocytic anemia with leucopenia. There was no increase in serum bilirubin or urobilinuria. The bone marrow showed megaloblastic hyperplasia. The absorption of glucose, fat, and iodide from the intestine was normal. Gastric acidity was normal after a test meal. Indirect evidence for the presence of the intrinsic factor in the gastric juice was furnished by the fact that the reticulocytes rose and the blood levels improved on a diet rich in Castle's extrinsic factor. However, complete alleviation of the gastro-intestinal symptoms and disappearance of the anemia only occurred after the dietary regime had been supplemented with intensive parenteral liver extract therapy. Except for mild relapses of gastro-intestinal symptoms when he has broken away from his therapeutic regime, the patient has remained clinically well, with a normal blood picture for two years following discharge from the hospital.

Case 50. R. J., male, aged 35, waiter.

History: This patient was admitted to the Hospital of The Rockefeller Institute on February 4, 1937, complaining of abdominal cramps, loss of weight, and weakness. He had enjoyed good health until 1922 when he began to suffer from epigastric pain, coming on 1–2 hours after meals and relieved by soda. After a short period of hospital treatment he was much improved and remained asymptomatic until 1930 when he suddenly awoke with excruciating abdominal pain. He was taken to the Bellevue Hospital where an emergency operation was performed for a perforated peptic ulcer; a gastro-jejunostomy was done at the time. Following this operation he still had mild epigastric distress and was troubled with abdominal distention and borborygmi. Eighteen months later he suddenly had a gross hematemesis. He was again admitted to the Bellevue Hospital and given a strict diet. X-rays revealed no definite ulceration. He then remained fairly well for 2 years before he suffered a second hematemesis. On this occasion he was admitted to another hospital where x-rays were interpreted as showing a duodenal ulcer. Coincident with this hematemesis the patient began belching very foul gas having a fecal odor. At operation no ulcer was found but abdominal adhesions were broken up. Following the operation the patient was worse with marked borborygmi, flatulence, diarrhea, anorexia, and loss of strength and weight. At times his stools would contain food which he had eaten only 2–3 hours before. Because red meat did not agree with him, he eliminated this from his diet. However, he continued to take a fairly well balanced diet with broiled liver at least 4 times a week in addition to fresh vegetables and fruit. Between periods of anorexia, his appetite was at times voracious, yet he steadily lost weight. On one occasion gas that he eructated while lighting a cigarette caught fire and singed his mustache. After this episode he kept his face clean-shaven.

The patient lost ground slowly over the next 18 months until December, 1936, when he was forced to stop work. Two weeks before admission to the Rockefeller Hospital the diarrhea gave way to constipation and the eructation of gas decreased. Although pallor was never marked, a definite macrocytic anemia was noted at the New York Hospital and it was for further study of this anemia that the patient was transferred to the Rockefeller Hospital. He had at no time suffered from sore tongue or neurologic symptoms.

Physical Examination: The patient was a small, emaciated, moderately pale man who had obviously lost much weight. The skin was slightly atrophic and drawn tight over the bony eminences of the body. The conjunctivae were somewhat pale; the sclerae were clear with no suggestion of icterus. The teeth were in fair shape; the oral mucosa and tongue appeared normal. There was no enlargement of the lymph nodes. The thyroid was not enlarged. The heart and lungs were normal. Blood pressure 115/80. The abdomen was rounded and distended with gas; it was extremely tympanitic. Scars from previous operations were in evidence. There was definite tenderness over the mid-epigastrium, more marked on rebound, and an increased resistance to palpation over the right side of the abdomen. The edge of the liver, sharp, smooth, and slightly tender, was palpable 2 to 3 cm. below the right costal margin; no other viscera or masses were felt. There was slight tenderness on rectal examination. Definite clubbing of the fingers and toes and slight acrocyanosis were noted. Neurologic examination was normal.

Laboratory Findings: Blood Count: R. B. C. 3,620,000, Hgb. 100%, W. B. C. 4,400 with normal differential count. M. C. V. $119\mu^3$, C. I. 1.38, icterus index 3. Platelets 310,000. The blood smear showed a striking uniformity in the size of the erythrocytes with a distinct tendency to macrocytosis but little poikilocytosis.

Urine: Normal.

Stools: Pale, yellow with many fat globules, fatty acid crystals, and starch granules; no parasites or ova; guaiac test negative.

Gastric Analysis: Showed a large amount of material in the fasting stomach, yellowish-gray in appearance, with a mixed yeasty and fecal odor. This material contained some free HCl. No typical gastric juice was obtained. A subsequent gastric analysis several weeks later showed typical fasting contents with free HCl of 65°.

Liver Function Tests: 1) Bilirubin excretion test:—Original plasma bilirubin level 0.6 mg. per 100 cc.; no retention of injected bilirubin after 4 hours. 2) Sodium benzoate test (2 separate occasions):—2.969 Gm. of benzoic acid and 2.58 Gm. benzoic acid excreted in 4 hours, slightly subnormal values. 3) Galactose tolerance test: trace of galactose excreted during the 4-hour period, a normal result.

Tests of intestinal absorption: 1) Glucose tolerance test: The patient was given 54.0 Gm. glucose by mouth (1.25 Gm. per Kg. body weight); the blood sugar rose from fasting level of 70 mg. to 125 mg. per 100 cc. at the end of one hour, pointing to fairly good absorption of glucose from the intestine. The actual figures for the test were as follows:

Specimen	*Blood Sugar*
Fasting	70 mg. per 100 cc.
½ hour after glucose	112 mg. per 100 cc.
1 hour after glucose	125 mg. per 100 cc.
2 hours after glucose	75 mg. per 100 cc.
3 hours after glucose	55 mg. per 100 cc.
4 hours after glucose	53 mg. per 100 cc.

2) Fat absorption test: After the ingestion of a fat meal furnishing 2 Gm. fat per kilo body weight, the patient showed a delayed but fairly good rise in the blood fat level:

Specimen	*Total plasma lipid carbon*
Fasting	250 mg. per 100 cc.
2 hours after fat meal	257 mg. per 100 cc.
4 hours after fat meal	448 mg. per 100 cc.
6 hours after fat meal	258 mg. per 100 cc.

3) Ascorbic acid absorption test: The patient showed a good response to the oral administration of 250 mg. ascorbic acid a day as measured by the increase in the 24 hour urinary output of ascorbic acid.

Fluoroscopy and X-rays of the gastro-intestinal tract showed a normal esophagus. The stomach was long and filled in its lower portion; when the barium reached a certain level, it appeared to spill out through a stoma into the jejunum. After considerable barium had passed into the small intestine, there appeared to be some spilling of barium into the colon although the point of communication could not be made out. X-rays confirmed the fluoroscopic findings and also showed some barium passing through the pylorus into the duodenum. A barium enema revealed a redundant relaxed colon which filled slowly; the barium did not pass beyond the middle of the transverse colon until pressure was exerted over the abdomen. Then the barium appeared to pass both into the rest of the colon and into the stomach. Previous x-rays taken at the New York Hospital had definitely established the presence of a gastro-jejuno-colic fistula.

Course: 1) After the diagnostic studies had been completed, the patient was given a diet for sprue, supplemented with autolyzed yeast, 12 Gm. a day. There was no definite improvement on this regime. 2) Therapy was changed from autolyzed yeast to liver extract, 12 Gm. a day (the amount of extract derived from 300 Gm. whole liver). The patient became more uncomfortable with abdominal distention and pain so that after a short trial the diet and liver extract had to be abandoned. His blood showed no change. It was felt at this time that a recurrence of the gastric ulcer or a jejunal ulcer might be responsible for some of his symptoms. 3) He was therefore placed upon the Meulengracht diet for bleeding ulcer (Meulengracht, 1936) with alkaline powders, and intravenous injections of liver extract were started. On this regime he had less abdominal discomfort but passed 3–4 loose stools a day and his blood count failed to improve materially. 4) Previous therapy was discontinued and the sprue diet resumed; the patient was also given intramuscular injections of calcium gluconate. Abdominal discomfort persisted until desiccated hog stomach, 20 Gm. a day, was started. This addition appeared to relieve the abdominal discomfort and to regulate the bowels. However, his blood count fell until the R. B. C. reached 2,980,000 and Hgb. 70%. Intramuscular injections of concentrated liver extract[4] (1 cc. derived from 100 Gm. whole liver) were then given and the patient was discharged from the hospital on August 5, 1937 to return to the out-patient clinic twice weekly for further injections of liver extract and calcium gluconate. He followed the diet for sprue as well as he could at home. Although he continued to have gaseous distention, foul eructations, and mild diarrhea, his general condition and blood count showed distinct improvement.

September 14: Wgt. 44.4 Kg., a gain of 8.0 Kg. from the lowest point during his hospital stay. R. B. C. 3,830,000, Hgb. 83%, M. C. V. 103μ^3, C. I. 1.09.

October 18: Wgt. 45.5 Kg. November 29: Diarrhea had increased; weight 42.1 Kg.

Because of the persistence of the gastro-intestinal symptoms and the recent loss of weight, the patient was admitted to the New York Hospital on December 1 for

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[4] The preparation used was Concentrated Liver Extract, Lederle.

TABLE I

CASE NO.	AUTHOR	YEAR	SEX	AGE	GLOSSITIS	G-I SYMPTOMS	ICTERUS	R. B. C.	HGB	C. I.	MACROCYTOSIS	FREE HCl*	INTESTINAL LESION	TREATMENT	RESULT†
								millions	*per cent*						
1	Wallis and Warfvinge	1893	F	75		+	+	1.40	40	1.43	+	0^{m}	4 non-tbc. strictures in lower jejunum and ileum		Died, autopsy
2	Faber	1895	F	27		+		0.60	20	1.66	+		2 non-tbc. strictures of jejunum		Died, autopsy
3	Holst	1896	F	49		+	+	0.48	20	2.08	+		14 non-tbc. strictures of ileum		Died, autopsy
4	Borchgrevink	1898	M	59		+		Severe anemia					4 tbc. intestinal strictures	Milk diet	Temp. improved; died, autopsy
5	Barker and Hunter	1900	M	28		+		1.00	20	1.00	+	$+^{v}$	Traumatic stricture of jejunum		Died, autopsy
6	Gluzinski	1911	F	44		+	+	1.12	31	1.39	+	0^{m}	Multiple constricting tbc. ulcers of jejunum and ileum		Died, autopsy
7	Bretschneider	1911	M	27		+		0.89			+		Multiple tbc. strictures of small intestine		Died, autopsy
8	A. Schmidt	1914	?					Severe pernicious anemia					Stricture of small intestine		Died, autopsy
9	A. Schmidt	1914	?					Severe pernicious anemia					Stricture of small intestine		Died, autopsy
10	Meulengracht	1921	F	38		+	+	2.70	50	0.93	+	0^{m}	Fibrous stricture in upper colon		Died, autopsy
11	Meulengracht	1921	F	56	+	+	+	1.70	39	1.15	+	0^{m}	Fibrous stricture of lower ileum	Arsenic and kefir milk	Temp. improved; died, autopsy
12	Meulengracht	1922	F	64		+	0	1.34	40	1.50	+	0^{m}	3 tbc. strictures of small intestine	Operation	Died, autopsy (V)
13	Szczepanski	1923	F	40				Typical pernicious anemia					Tbc. mesenteric adenitis and intestinal strictures		Died, autopsy
14	Meulengracht	1922 1929	M	52	+	+	+	1.46	45	1.52	+	0^{m}	Operative anastomosis and p-o strictures	(a) arsenic and kefir milk (b) liver therapy	Remissions and relapses Cure (VI)
15	Seyderhelm	1924 1927	F	?				Pernicious anemia					Stricture of small intestine	Operation	Cure (V)
16	Kretz	1925	F	27	+	+		3.73	78	1.04	+		Tbc. stricture of cecum		Died, autopsy

17	Wischmann and Zinsser	1926	F	22	+	+		0.95	26	1.37	+	0^m	Stricture of ascending colon		Died
18	Zadek	1926	F	44	+	0	+	0.81	19	1.17	+	0	Tbc. ulcer and stricture of lower ileum		Died, autopsy
19	Zadek	1926	M	65	0	0	0	3.64	79	1.06	0	0	Fibrous tbc. ulcer of transverse colon		Died, autopsy
20	Deutsch	1926	F	13		+	+	1.75	40	1.14	+	$+^m$	Small intestine constricted by tbc. adhesions (x-ray)	Carbohydrate and milk diet	Improved
21	W. Schmidt	1927	M	71	+	+		0.88	28	1.75	+	0	Adhesions constricting descending colon		Died, autopsy
22	Hartmann	1929	F	67		+		2.26	50	1.13	+	0^m	Foreign body in abdominal cavity with intestinal anastomosis and blind cul-de-sac		Died, autopsy
23	Meulengracht	1929	F	63	+	+		2.60	60	1.17	+	0^m	3 strictures of lower ileum; prob. tbc.	Operation	Died, autopsy (V)
24	Little, Zerfas, and Trusler	1929	M	28	+	+	+	1.30			+	$+^m$	2 operative entero-enterostomies and fecal fistulas	(a) Operation (b) Liver therapy	Improved (VII) Temp. improved. Later died, autopsy
25	Taylor	1930	F	54	+	+	+	1.20	42	1.75	+	0^h	Multiple diverticula of intestine	(a) Liver therapy (b) Operation	Improved Died, autopsy (VII)
26	Becker	1930	F	65	+			1.87	48	1.28		+	Ca. of hepatic flexure with stricture	Liver therapy	Died, autopsy (VI)
27	de Rivas	1930	M	55		+		Sprue anemia					Gastro-colostomy		Died, autopsy
28	Scherer	1930	F	44	+	+	+	2.04	44	1.08	+	0^m	Tbc. stricture of lower ileum	Operation	Improved (V)
29	Strandell	1931	F	56		+		0.90	30	1.69	+	0^m	Multiple strictures of ileum		Died, autopsy
30	Strandell	1931	F	48	+	+	+	1.70	48	1.41	+		Adhesions constricting transverse colon		Died, autopsy
31	Castle, Heath and Strauss	1931	M	41	+	+	+	0.91	35	1.90	+	$+^h$	Multiple operative anastomoses and p-o fistulae	Liver therapy	Improved (VI)
32	Narbeshuber	1931	F	30	+	+	+	2.16	50	1.16	+	$+^m$	Entero-enterostomy and ileocolostomy to circumvent 3 tbc. strictures	Liver therapy	Cure (VI)
33	Fairley and Kilner	1931	M	42	+	+	0	3.60	65	0.90	+	$+^f$	Gastro-jejuno-colic fistula	(a) Liver therapy (b) Operation	Improved Cure (VII)
34	Fairley and Kilner	1931	M	49	+	+	0	3.13	77	1.22	+	$+^f$	Ca of stomach; gastro-jejuno-colic fistula	(a) Liver therapy (b) Operation	Improved Died (VII)
35	Fairley and Kilner	1931	M	58	+	+	0	3.60	68	0.94	+		Gastro-jejuno-colic fistula	Liver therapy	Died, autopsy (VI)

TABLE I—*Concluded*

CASE NO.	AUTHOR	YEAR	SEX	AGE	GLOSSITIS	G- SYMPTOMS	ICTERUS	R. B. C.	HGB	C. I.	MACROCYTOSIS	FREE HCl*	INTESTINAL LESION	TREATMENT	RESULT†
								millions	*per cent*						
36	Bremer	1932	?					Pernicious anemia					Strictures of small intestine		Died, autopsy
37	Bremer	1932	?					Pernicious anemia					Strictures of small intestine		Died, autopsy
38	Hurst	1933	F	30	+	+	+	2.20	54	1.23	+	+	P-o strictures and operative anastomoses	Operation	Died (V)
39	Emile-Weil, Stieffel, and Bousser	1933	M	65	0	+	+	1.10	40	1.82	+	$+^{h}$	Adhesions obstructing ascending and trans. colon	Liver therapy	Died, autopsy (VI)
40	Schlesinger	1933	F	54	+	+	+	1.53	37	1.21	+	$+^{m}$	Operative entero-enterostomy; some obstruction	Liver therapy	Improved (VI)
41	Strauss Castle	1934 1937	M	8	+	+		1.27	24	0.95	+	+	2 operative entero-enterostomies	Liver therapy	Cure (VI)
42	Christopher	1935	M	17		+		1.89	45	1.19			Operative jejuno-colostomy and obstructed ileo-colostomy	Operation	Cure (V)
43	Sturgis	1936	F	42	+	+	0	2.97	65	1.09	+	$+^{h}$	Ileo-colostomy with long loop	Liver therapy	Cure (VI)
44	Hawksley and Meulengracht	1936	F	49	+	+	0	1.96	45	1.15	+	0^{m}	Active tbc. with multiple strictures	(a) Liver therapy (b) Operation	Slightly improved Died, autopsy (VII)
45	Minot Richardson	1936 1938	M	30	+	+	+	2.90	60	1.04	+	$+^{m}$	Entero-enterostomy short-circuiting nearly ⅔ small intestine	(a) Liver therapy (b) Operation	Improved Cure (VII)
46	Butt and Watkins	1936	F	49		+		3.19	88	1.37	+	0	Subacute ileitis and colitis with some obstruction	Operation	Cure (V)
47	Butt and Watkins	1936	M	55		+		2.93	81	1.38	+	0	Ileitis and colitis with obstruction	(a) Liver therapy (b) Operation	Improved Died, autopsy (VII)
48	Butt and Watkins	1936	F	60	+	+		3.25	89	1.37	+	0	Multiple fecal fistulae	Operation	Died (V)
49	Butt and Watkins	1936a	M	55		+		3.59	84	1.16	+	+	Chronic ileitis with partial obstruction	(a) Operation	Temp. improved; relapse (VII)
	Barker and Hummel	1938b			+	+	0	2.35	69	1.47	+	$+^{m}$	Operative ileo-colostomy	(b) Liver therapy	Cure
50	Barker and Hummel	1938	M	35	0	+	0	3.62	100	1.38	+	$+^{f}$	Gastro-jejuno-colic fistula	(a) Liver therapy (b) Operation	Temp. improved Died, autopsy (VII)
51	Bethell	1938	M	29	0	+	+	2.48	60	1.20	+	$+^{h}$	4 strictures of lower ileum	(a) Liver therapy (b) Vegex therapy (c) Operation	Slightly improved Much improved (VII) Temporary cure

* The letters after the symbols + or 0 may be interpreted as follows: "f" = fasting; "m" = after test meal; "h" = after histamine; "v" = vomitus.

† The Roman numerals V, VI, VII after certain cases refer to Tables V, VI, VII in which the therapy and result are given in more detail.

operative correction of the gastro-jejuno-colic fistula. At this time his blood picture was as follows: R. B. C. 3,700,000, Hgb. 85%, W. B. C. 9,000. On December 8 the gastro-jejuno-colic fistula was repaired and the normal continuity of the gastro-intestinal tract was restored. Following the operation patient did well at first, but still complained of epigastric pain from time to time. On January 18, 1938 his stools became tarry and the red blood cell count had dropped to 2,700,000. Gastric lavage and x-rays showed considerable gastric retention. The patient was then given several transfusions and the abdomen was explored on February 15. Since the original ulcer was found to be perforating into the pancreas, a gastric resection by the Polya technique was carried out but the patient developed a duodenal fistula, generalized peritonitis and bronchopneumonia of which he died on February 24, 9 days after the second operation.

Autopsy[5] revealed unhealed wounds of resection of the pylorus with gastro-enterostomy and entero-enterostomy; a communication between the duodenum and the peritoneal cavity at the point of closure of the duodenum; generalized peritonitis; an ulcer of the transverse colon at the site of closure of the jejuno-colic fistula; hydrothorax, left; extensive atelectasis of the lower lobes of both lungs; abscesses in the kidneys; petechiae and ecchymoses of the skin, pericardium, pleura, and mucosa of the stomach and bladder. The liver was normal to both gross and microscopic examination. The vertebral bone-marrow was grossly quite red and sections showed a moderate increase of cellularity with a very definite decrease in mature forms of both the white cell and the red cell series. There were practically no normoblasts and very few leucocytes or adult myelocytes present. Young myelocytes and crythroblasts were abundant in addition to a number of cells which were interpreted as megaloblasts.

Summary: A 35 year old white waiter had suffered from symptoms of a gastric ulcer since 1922. Eight years after the onset of symptoms the ulcer perforated. A gastro-enterostomy was performed at this time. During the next 4 years he suffered two large hematemeses and coincident with the second of these he developed fecal belching. In 1935 a second operation was performed for release of adhesions. Subsequently he developed anorexia, flatulence, and diarrhea, and there was progressive loss of strength and weight. The principal physical findings on admission to the hospital in 1936 were emaciation, slight pallor, gaseous abdominal distention, epigastric tenderness, slight enlargement of the liver, and clubbing of the fingers and toes. There were no neurologic abnormalities. A slight anemia, macrocytic in type, with leucopenia was noted. Free hydrochloric acid was present in the gastric juice. Roentgenographic study of the gastro-intestinal tract revealed a gastro-jejuno-colic fistula. The absorption of glucose, fat, and ascorbic acid from the intestinal tract was surprisingly good. Although the patient at first gained weight on a therapeutic regime consisting of a diet for sprue and intramuscular injections of liver extract, the gastro-intestinal symptoms and the macrocytic anemia persisted. For these reasons an operation was performed and the gastro-jejuno-colic fistula was repaired. The patient improved temporarily but then developed pyloric obstruction and melena, necessitating a second operation two months later. Following this he died of peritonitis. At autopsy the bone marrow was found to be hyperplastic with immature cells of both the erythroid and the myeloid series predominating.

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[5] We are indebted to Dr. William Andrus of the Cornell University Medical School for permission to include details of the patient's course at the New York Hospital and the results of the post-mortem examination.

ANALYSIS OF 51 CASES

The principal clinical and pathologic features of 51 cases (49 from the literature and 2 of the authors) of macrocytic anemia associated with intestinal stricture or anastomosis are presented in chronological order in Table I. The frequency with which certain symptoms, hematologic changes, and abnormalities of gastric secretion occurred are summarized in Table II.

TABLE II

Symptomatology and laboratory data in 51 cases

	PRESENT	ABSENT	NOT MENTIONED
Gastro-intestinal symptoms	42	2	7
Glossitis	25	4	22
Icterus	18	10	23
C. N. S. involvement	10	12	29
Anemia	51		
Macrocytosis	39	1	11*
Hyperchromia	32	10	9
Leucopenia	30	6	15
Free HCl in gastric juice†	17	19	15

* Anemia spoken of simply as "pernicious anemia" in these cases; no specific mention of macrocytosis.

† See Table III for further analysis.

INCIDENCE AND SYMPTOMATOLOGY

Age and Sex. The ages of the patients ranged from 8 years to 75 years in 46 cases; in the other 5 cases the age was not given. Thirteen of the 46 patients, or 28 per cent, were less than 30 years of age when they developed their anemia, and 3 of the patients were under 20. Thus the incidence of this form of anemia in young people is considerably higher than is the case in a comparable series of patients with true "idiopathic" pernicious anemia.

The sex was specified in 47 cases, of which 27 were females and 20 males.

Gastro-Intestinal Symptoms. Disturbances referable to the gastro-intestinal tract were prominent in 42 of the 44 cases, where symptomatology was given. The symptoms were essentially those of partial intestinal obstruction: diarrhea or intermittent diarrhea and constipation, distention, abdominal cramps, and less frequently nausea and vomiting. Visible intestinal peristalsis and audible borborygmi were frequently noted along with abdominal fullness and tenderness, especially in the right lower quadrant. Fecal belching was a feature in the cases of gastro-jejuno-colic fistula. Marked loss of weight, secondary to the gastro-intestinal disturbances, was the rule.

Glossitis. Soreness of the tongue, with or without papillary atrophy, was present in 25 cases, absent in 4, and not mentioned in 22. The incidence of some form of glossitis in this form of anemia is comparable to that in true pernicious anemia and sprue.

Icterus. The presence or absence of icterus was mentioned in 28 cases; 18 of these showed clinical icterus, confirmed in many instances by a positive van den Bergh reaction or an elevated icterus index. In one case (no. 20) the level of the serum bilirubin was 6.0 mg. per 100 cc.; in another case (no. 45) the icterus index was 60.

Neurologic Symptoms. Symptoms and signs of involvement of the nervous system were not so common in this group of patients as in patients with true pernicious anemia. In only 4 cases (nos. 24, 25, 35 and 38) was objective evidence of disease of the nervous system described; 3 of these cases presented signs of combined degeneration of the spinal cord whereas one (no. 35) had a peripheral neuritis. Six other patients complained of paresthesias in the extremities, but showed no objective neurologic changes. In 12 cases there was said to be no involvement of the nervous system whatsoever; no mention of neurologic manifestations was made in the other 29 cases.

LABORATORY DATA

Blood Picture. Anemia of the pernicious type was present in all 51 cases. Actual figures for erythrocyte and hemoglobin levels were given in 44 cases. The red blood cells varied from 480,000 to 3,730,000 per cubic millimeter; in 8 cases the erythrocyte count was below 1,000,000. The values for hemoglobin varied from 19 per cent to 100 per cent.

The occurrence of macrocytosis was mentioned specifically in 39 cases; one patient (no. 19) showed a normocytic anemia, while in the remaining 11 cases the cell size was not specified. However, macrocytosis may be assumed in the latter since the blood in each instance was said to present the typical picture of pernicious anemia. That the macrocytosis was often pronounced is apparent from the figures for the mean diameter of the erythrocytes or the mean corpuscular volume which were included in certain of the case reports. In one case, a mean erythrocyte diameter of 9.1 microns is recorded, while in another, cells measuring up to 17 microns in diameter were noted. A mean corpuscular volume of over 120 cubic microns was not unusual.

Hyperchromia was present in 32 of the 42 cases in which figures are available. The color index varied from 0.90 to 2.08 with values above 1.30 in 18 of the cases.

As in true pernicious anemia leucopenia was an extremely common finding. In 30 of 36 cases in which the leucocyte count was recorded, values of 6,000 white blood cells per cubic millimeter or lower were reported, and in 23 of these cases the leucocyte count was 5,000 or below.

Gastric Analysis. The results of gastric analysis were given in 36 cases, of which 17 (47 per cent) showed free hydrochloric acid in the gastric juice. This percentage would probably be considerably higher had more of the patients who showed achlorhydria after a test meal received histamine. In Table III the presence or absence of free hydrochloric acid in the gastric juice is tabulated with respect to the type of specimen examined.

The presence of free hydrochloric acid in the gastric juice of such a large percentage of these cases sharply demarcates this form of macrocytic anemia from true pernicious anemia where complete achlorhydria, even after stimulation with histamine, is the rule. The results of the gastric analyses in this group of patients are comparable to those obtained in patients with sprue (Miller and Barker).

Direct tests for the presence of Castle's intrinsic factor in the gastric juice were carried out in 2 cases of this series by means of gastric juice feeding experiments. In one (no. 40) conclusive proof of the presence of intrinsic factor was furnished, whereas in the other (no. 31) no intrinsic factor could be demonstrated. The excellent hematologic response of our first case (no. 49) and of case no. 51 to a diet rich in Castle's extrinsic factor was regarded as indirect evidence for the presence of intrinsic factor in the gastric juice of these patients.

Studies of Intestinal Absorption. It is unfortunate that intestinal absorption was studied in so few of these cases. The presence of fat in the stools was noted in 9 cases, whereas in 2 cases (nos. 5 and 51) the feces were said to contain no fat. In the authors' 2 cases, the absorption of fat from the intestine was studied by a more accurate method described in detail elsewhere (Barker and Rhoads), i.e., the blood-lipid curve following the ingestion of a fat meal. Both patients gave essentially normal absorption curves which stand out in sharp contrast to the perfectly flat curves observed in patients with active sprue.

TABLE III

Gastric analysis in 36 cases

SPECIMEN OF GASTRIC JUICE	FREE HYDROCHLORIC ACID	
	Present	Absent
Fasting	3	
After test meal	6	12
After histamine	4	1
Vomitus	1	
Not specified	3	6
Totals	17	19

The absorption of glucose was studied by means of the oral glucose tolerance test in 4 cases (nos. 28, 33, 49 and 50). Although the first of these gave a flat blood-sugar curve comparable to that characteristic of sprue (Fairley; Hanes and McBride), the other 3 showed practically normal curves suggesting that glucose was readily absorbed in these cases.

Other observations upon the question of intestinal absorption have been included in the authors' two case reports. Suffice it to say that the available data, scanty though they are, strongly suggest that defective intestinal absorption alone cannot explain the clinical picture presented by this group of patients.

PATHOLOGIC ANATOMY

Pathologic Diagnosis. Thirty-six of the 51 patients of this group died, and the diagnosis of intestinal stricture or anastomosis was established at autopsy in 31 of the 36; of the other 5 cases, the diagnosis was made at operation in 4, and by roentgen examination of the intestinal tract in one. Fifteen patients were alive at the time their cases were reported; in 13 of these the presence of a stricture or anastomosis was ascertained at operation; in the other 2 the diagnosis was made radiographically.

TABLE IV

Gastro-intestinal lesions

Strictures:		
Small intestine:		
Tuberculous	11	
Non-tuberculous	6	
Not specified	8	
Total		25
Large intestine:		
Tuberculous	1	
Non-tuberculous	2	
Carcinoma	1	
Post-operative	1	
Not specified	2	
Total		7
Anastomoses:		
Entero-enterostomy or entero-colostomy	13	
Gastro-jejuno-colic fistula	4	
Gastro-colic fistula	1	
Total		18
Diverticulosis		1
Total		51

Lesions. The pathologic findings of principal interest were limited to the gastro-intestinal tract and the bone-marrow.

1: *Gastro-intestinal tract:* In Table IV the various types of gastro-intestinal lesions are summarized. Strictures of the intestine, either single or multiple, were present in 32 cases, 25 in the small intestine and 7 in the colon. The majority of the strictures of the small intestine were located in the ileum; the jejunum was involved in a few instances. Of the strictures of the small intestines, 11 were definitely tuberculous in origin, 6 were said to be non-tuberculous, and in 8 the etiology was not specified. Certain of the non-tuberculous strictures were due to a non-specific terminal ileitis, as for example in 3 of the cases reported by Butt and Watkins. Incidently these authors described a macrocytic blood picture in several other cases of constrictive ileitis which are not included in the present review because the macrocytosis was not accompanied by definite anemia.

Anastomoses of one sort or another formed the basic abnormality in 18 cases. In 13 of these cases the anastomosis was either an entero-enterostomy or an entero-colostomy resulting from a surgical procedure which had been carried out for the relief of intestinal obstruction. Most of these operations had been performed to circumvent strictures or constricting adhesions which had developed subsequent to some previous operation, frequently appendectomy. In 2 of the 13 cases the anastomoses were complicated by fecal fistulas. 4 of the remaining 5 patients had gastro-jejuno-colic fistulas: in 3 of these the fistula developed after gastro-enterostomy for pyloric obstruction, whereas carcinoma of the stomach was responsible for the fistula in the fourth case. De Rivas' case (no. 27) was found at autopsy to have a gastro-colic fistula which had been created by error instead of the intended gastro-jejunostomy.

2. *Bone marrow:* Data on the appearance of the bone-marrow were furnished in 11 cases: 10 from post-mortem examination and one from sternal biopsy. Of the first 10, 9 showed a dark red hyperplastic marrow further described as "raspberry-like" in one case and as "megaloblastic" in three cases; in one case (no. 39) the marrow was said to be fatty and aplastic. The authors' first case (no. 49) is the only one in which the marrow was studied by sternal biopsy before and after treatment with liver extract. The striking change from megaloblastic hyperplasia to an essentially normal marrow picture has already been described. There can be little doubt from the pathologic descriptions of these 11 cases that the bone-marrow picture in stricture

anemia is very similar to, if not identical with, that seen in true pernicious anemia and in the macrocytic anemia of sprue.

TREATMENT

The patients of this series have been divided into four groups according to the form of treatment they received: I, patients who received neither operative treatment nor liver therapy; II, patients treated by surgical means alone; III, patients treated with some form of liver therapy alone; IV, patients treated by both surgical means and liver therapy. Such a grouping serves to facilitate an evaluation of the results that have been obtained with different forms of therapy.

Group I. Patients who received neither operative treatment nor liver therapy: This group is made up of 23 patients who received neither liver therapy nor operative treatment as far as can be ascertained from the case reports. It is only fair to state that a number of the cases included in this group were reported very briefly and that therapy was not mentioned at all in several of these reports. Thus we cannot be certain that some specific form of therapy was not attempted in these cases. However, the majority of the cases comprising this group were observed before the advent of liver therapy.

Most of these patients simply received supportive treatment and succumbed to their illness or the complications thereof shortly after they first came under observation. Twenty-two of the twenty-three patients died and twenty-one were autopsied.

In view of the successful use of a milk diet in the treatment of certain cases of true pernicious anemia before the liver era (Barker and Sprunt), it is of interest that three patients of this group (Cases 4, 11 and 20) who were placed upon milk diets were distinctly benefited by such a dietary regime. All three showed remissions in the macrocytic anemia and, although two cases subsequently relapsed and died, the third patient (no. 20) was gaining weight and doing well at the time of the case report. In this case the blood picture had shown a complete change from the original macrocytosis and hyperchromia to a distinct microcytosis and hypochromia: before the milk diet, R. B. C. 1,750,000 with Hgb. 40%; after treatment, R. B. C. 4,620,000 with Hgb. 40%.

Two other cases were treated with colonic irrigations and arsenic;

one (no. 27) showed no improvement and died very shortly; the other (no. 6) experienced repeated remissions and relapses over a period of several years before suffering a fatal relapse.

Group II. Patients treated by surgical means alone (Table V): Of the nine patients who were subjected to some form of surgical procedure, five died so soon after operation that no conclusions can be drawn as to the effect of the operation on the blood picture. The four patients who survived showed striking symptomatic and hematologic improvement following operation. One patient (no. 15) was said

TABLE V

Patients treated by surgical means alone

CASE NO.	LESION	OPERATION	RESULT
5	Stricture of jejunum	Entero-enterostomy	Died 2 days p-o
12	3 tuberculous strictures of small intestine	Resection of strictures	Died 12 hours p-o
15	Stricture of small intestine	Resection of stricture	Well 3 years p-o
23	3 strictures of lower ileum	Resection of 2 strictures	Died of complications, 5 weeks p-o; 3rd stricture found
28	Tbc. stricture of lower ileum	Ileo-colostomy	Marked blood rise, symptomatic improvement, and weight gain. Short follow-up
38	Post-operative strictures and operative anastomosis	Cecostomy	Died 3 weeks p-o
42	Operative jejuno-colostomy	Anastomosis taken down	Blood rose to normal; gain 45 lbs. in 3 months
46	Terminal ileitis with stricture	Ileo-colostomy	Anemia cleared up; no follow-up
48	Multiple fecal fistulas	Repair of fistulas; resection of sigmoid	Died p-o

to have remained perfectly well for three years after the resection of a stricture of the small intestine, while another (no. 42) with a jejuno-colostomy showed a remarkable gain in weight and complete disappearance of the anemia within three months after the anastomosis had been closed.

In each of the other two cases (nos. 28 and 46) an ileo-colostomy was performed to circumvent strictures of the terminal ileum. The immediate results were excellent in both instances since the anemia cleared up completely. However, the shortness of the follow-up periods in these two cases gives little or no assurance that they did not later relapse in the manner so graphically illustrated by the authors' first case (no. 49—see under Group IV). When one is considering the advisability of an anastomotic operation in the treatnent of intestinal stricture, it is well to recall that in 13 of the 51 cases of macrocytic anemia comprising the total series the anemia first developed *after* such operations had been performed to circumvent strictures.

Group III. Patients treated with liver alone (Table VI): This group consists of nine cases in which the anemia was treated without operation but with some form of liver therapy. It is to be noted that six patients in the group first developed macrocytic anemia after undergoing anastomotic operations which were designed originally to circumvent strictures.

Three of the patients in this group of nine died without evidence of improvement: case 26 died of carcinoma of the colon; case 39 of infection—the bone marrow was said to have been aplastic in this case; and case 32, a patient with a gastro-jejuno-colic fistula who was desperately ill, died the third day after the institution of liver therapy.

The remaining six patients showed striking improvement on liver therapy and maintained excellent health during the subsequent periods of observation. Three patients gave satisfactory responses to the oral administration of liver preparations; such responses may be accepted as evidence that there was no marked disturbance in intestinal absorption in these cases. It is of interest that one of these three patients (no. 31) had failed to improve on a preliminary course of 200 grams of raw beef a day, an excellent source of Castle's extrinsic factor. It was conclusively demonstrated that the gastric juice of this same patient contained no intrinsic factor. A fourth patient (no. 40) was first

TABLE VI

Patients treated with liver alone

CASE NO.	LESION	TREATMENT	RESULT
14	Post-operative strictures and anastomosis	(a) Arsenic, kefir milk, transfusions (b) Oral liver and L. E.	Remissions and exacerbations Well for one year; no further follow-up
26	Carcinoma with stricture of hepatic flexure	Oral liver and arsenic	Retic. rise but no blood rise; died 6 weeks later
31	Operative anastomoses and fecal fistulas	(a) 200 gm. raw beef q.d. (b) Oral L. E.	No improvement Prompt clinical improvement, retic. rise, and blood rise
32	Entero-enterostomy and ileo-colostomy	Whole liver and oral L. E.	Striking clinical improvement; blood returned to normal
35	Gastro-jejuno-colic fistula	Intramuscular L. E.	Died 3rd day
39	Post-operative adhesions constricting colon	Intramuscular L. E. and transfusions	Died of infection; aplastic bone marrow at autopsy
40	Operative anastomosis	(a) Intramuscular L. E. (b) Oral stomach preparation	Rapid rise in blood count Continued improvement; short follow-up
41	2 operative anastomoses	(a) Oral L. E. (b) Intramuscular L. E. 2 times a week	No response Complete remission; remained well during 3 year follow-up, on continued treatment
43	Operative ileo-colostomy	(a) Oral L. E., stomach yeast (b) Intensive intramuscular L. E. therapy	Little improvement Symptomatic and hematologic cure. Well during 1 year follow-up on continued treatment

treated parenterally with liver extract to which she responded well; her continued improvement after an oral preparation of desiccated stomach had been substituted for the parenterally administered liver extract was regarded as evidence that she was capable of absorbing the anti-anemic principle from her intestinal tract. The last two patients (nos. 41 and 43) failed to improve on oral liver preparations, yet subsequently developed complete remissions on intensive parenteral liver extract therapy. Two possible explanations may be offered for such a course of events: 1) that the patients did not absorb the anti-anemic principle from the intestinal tract, or 2) that they required larger amounts of the principle than was supplied by oral dosage used.

Group IV. Patients treated by both surgical means and liver therapy (Table VII). Ten patients were treated with both operation and liver therapy at some time in the course of their illness. The failure of the patient to obtain relief from the first form of therapy that was instituted led to the adoption of further therapeutic measures in each of these cases.

In seven cases an attempt was first of all made to treat the condition by medical means such as a diet for sprue, the oral administration of yeast preparations, or some form of liver therapy. The blood picture showed considerable improvement in five of these cases (nos. 25, 33, 34, 45 and 47), but the persistence of gastro-intestinal symptoms in three cases made surgical intervention imperative. All three patients died shortly after operation: case no. 25 of diverticulitis, no. 34 of carcinoma of the stomach, and no. 47 after resection of the colon. One patient (no. 33) improved in every respect on a diet for sprue, supplemented with oral liver extract but relapsed when he stopped taking the liver extract. An operation was then performed, the gastro-jejuno-colic fistula was repaired, and he made a complete recovery without the aid of further liver therapy. In case no. 45 the macrocytic anemia improved under parenteral liver extract therapy, but considerable jaundice persisted. The patient was therefore operated upon and a long-loop intestinal anastomosis was taken down; following this procedure the jaundice cleared and the patient remained perfectly well with a normal blood picture during the two year follow-up period without further liver therapy.

The other two cases treated first by medical measures (nos. 44 and 50) showed no definite improvement in the blood picture under liver therapy. In case 44 the reticulocytes rose after the administration

TABLE VII

Patients treated by both surgical means and liver therapy

CASE NO.	LESION	TREATMENT	RESULT
24	2 operative anastomoses and fecal fistulas	(a) Operation: anastomoses taken down (b) Oral L. E. (c) Stopped L. E. and later resumed (d) Stopped L. E. for 2nd time	Diarrhea ceased; anemia persisted Anemia cured Relapse and remission Developed combined system disease and died; autopsy showed obstruction from adhesions
25	Diverticulitis of intestine	(a) Oral L. E. (b) Operation: gastro-enterostomy and duodeno-enterostomy	Blood remission; vomiting persisted Died 2 days after 2nd operation
33	Gastro-jejuno-colic fistula	(a) Milk diet (b) Sprue diet and oral L. E. (c) Stopped L. E. (d) Operation: gastro-jejuno-colic fistula repaired	Temporary improvement Marked symptomatic and hematologic improvement Relapse Cure
34	Gastro-jejuno-colic fistula	(a) Sprue diet and oral L. E. (b) Operation: fistula repaired	Blood improved; symptoms persisted Died with carcinoma of stomach
44	10 Tbc. strictures of lower jejunum	(a) Oral stomach and liver preparations; parenteral L. E. (b) Operation: anastomosis to circumvent strictures	Mild retic. response but no blood rise Died soon afterwards with active tuberculosis
45	Operative anastomosis with long loop	(a) Oral L. E. (b) Intramuscular L. E. 2 times a week (c) Operation: anastomosis taken down	Temporary improvement; later relapse Blood rose to normal, jaundice persisted Complete cure without further liver—2 year follow-up

TABLE VII—*Continued*

CASE NO.	LESION	TREATMENT	RESULT
47	Ileo-colitis with strictures	(a) Parenteral L. E. (b) Resection of colon	Blood responded, diarrhea persisted Died post-operative
49	Terminal ileitis with strictures	(a) Operation: ileo-colostomy to circumvent strictures (b) Sprue diet, oral and parenteral L. E.	Temporary improvement in blood; later severe relapse Symptomatic and hematologic cure—2 year follow-up
50	Gastro-jejuno-colic fistula	(a) Sprue diet, Vegex, Ventriculin, oral and parenteral L. E. (b) Operation: fistula repaired (c) Operation: Polya gastro-enterostomy	Temporary weight gain; blood count variable; never entirely free of G-I symptoms Developed symptoms of pyloric obstruction Died 9 days later of complications
51	Post-operative adhesions constricting small intestine	(a) Operation: release of adhesions (b) Parenteral L. E. (c) Vegex orally (d) Operation: resection of 60 cm. of strictured ileum (e) Oral Vegex	Little change Moderate blood rise Marked improvement; gain of 40 lbs.; blood rose to normal; later relapse Blood rose to normal and remained so 8 months without liver or Vegex; then recurrence of G-I symptoms and anemia Moderate improvement

of the anti-anemic principle but there was no rise in the blood count. An anastomosis was then performed to circumvent ten tuberculous strictures of the lower jejunum but the patient died with active tuberculosis shortly afterwards. Case 50, the second of our two cases, presented a difficult therapeutic problem as symptoms of peptic ulcer complicated the clinical picture. Under various forms of medical therapy the patient temporarily gained weight but his erythrocyte count and hemoglobin level showed rather wide fluctuations and failed to return to normal. He was therefore subjected to operation, and the gastro-jejuno-colic fistula was taken down. Symptoms of pyloric obstruction and bleeding then developed, necessitating a second operation of the Polya type, and the patient died of complications nine days later.

In three cases (nos. 24, 49, and 51) the patients were first treated by surgical means. The failure of the operative treatment to relieve symptoms completely led to the subsequent institution of some form of liver therapy. The first of these patients (no. 24) underwent an extensive corrective operation for the repair of two intestinal anastomoses and several fecal fistulas. Diarrhea which had been troublesome before operation ceased, but the macrocytic anemia persisted until the patient had received liver extract by mouth. A blood remission then occurred and was maintained as long as the patient continued to take the liver extract. When he stopped taking liver extract the anemia recurred only to clear up again when liver therapy was resumed. The patient eventually stopped taking liver extract once more and developed a severe macrocytic anemia with combined degeneration of the spinal cord of which he died. This patient is of particular interest since the presence of free hydrochloric acid in his gastric juice would seem to rule out a coincidental "idiopathic" pernicious anemia.

The course of events in case 49, the first of our two cases, has been described in detail above. An anastomosis between the ileum and the transverse colon was first performed to circumvent the strictured terminal ileum. The mild macrocytic anemia present before operation temporarily disappeared after the anastomosis was performed, only to recur in a much more severe degree several months after operation. The relapse of the anemia was accompanied by diarrhea, flatulence, and glossitis. The anemia and gastro-intestinal symptoms cleared up entirely on a strict anti-sprue regime supplemented by parenteral liver extract and the patient has continued in excellent health for the past two years through strict adherence to the therapeutic program that was outlined for him.

Cast 51, treated alternately with operations and medical therapy, experienced repeated remissions and relapses as shown in the table. The development of fresh constricting adhesions was apparently responsible for the occurrence of the relapses.

From a study of this group of ten cases which were treated by both medical and surgical measures the complexity of the therapeutic problems which confront the physician becomes apparent at once. It is obvious that no single form of therapy will prove effective for all cases and that each patient presents an individual problem in therapy.

General Conclusions on Treatment: Certain conclusions may be drawn from this analysis of the results obtained with the various forms of therapy that have been employed in the treatment of "stricture anemia": 1) without either operation or liver therapy the prospect for improvement is very slight; 2) operations designed to correct the intestinal lesions are necessarily difficult procedures attended by a high operative mortality; 3) an operation successful in eliminating the mechanical abnormality may or may not be followed by complete cure; 4) the resection of a stricture or the restoration of the normal continuity of the bowel (in cases where an anastomosis is the offending lesion) is more likely to be attended by success than is an anastomotic operation designed to circumvent a stricture; 5) although liver therapy may bring about improvement in the blood picture, such therapy alone cannot be expected to relieve gastro-intestinal symptoms which are due to obstructive lesions in the intestinal tract; 6) surgical intervention is indicated in all cases with strictures and in young patients with anastomoses; preliminary liver therapy may serve to alleviate the anemia and thus reduce the operative risk in such cases; 7) liver therapy alone is indicated in older patients with well-functioning anastomoses where the operative risk is often extremely poor.

DISCUSSION

Two cases of macrocytic anemia in association with intestinal anastomoses have been reported in detail and 49 cases of a similar type of anemia occurring in patients with intestinal strictures or anastomoses have been collected from the literature. We have analyzed these cases from the standpoint of symptomatology, hematology, pathology, and treatment.

It is our impression that these cases do not represent a simple coincidence of an intestinal lesion and "idiopathic" pernicious anemia in most (if in any) of the patients of this group. Three points may be offered in support of this impression: the gastric juice of a large number of the patients contained free hydrochloric acid; in one case the intrinsic factor of Castle was present in the gastric juice in normal amounts as shown by direct test, whereas in two other cases indirect evidence suggested that the gastric juice contained the intrinsic factor; and finally, most important of all, in six cases the surgical correction of the intestinal abnormality was followed by disappearance of the anemia without the aid of liver therapy.

If, then, coincidental "idiopathic" pernicious anemia (due to the absence of the intrinsic factor) can be dismissed as an explanation for the anemia in many if not all of these cases, other possible etiologic mechanisms for the production of macrocytic anemia must be considered. These include: 1) deficient absorption of the hematopoietic principle such as occurs in sprue; 2) diets deficient in the extrinsic factor; 3) disease of the liver; 4) failure of interaction between the intrinsic and extrinsic factors in the gastro-intestinal tract or destruction of the hematopoietic principle through abnormal bacterial activity in the intestine; and 5) the absorption of hemotoxic substances from the intestinal tract. It is extremely difficult to place a given case of macrocytic anemia in any one of the above categories since the situation is usually a complicated one and a combination of two or more factors undoubtedly plays a rôle in the majority of the cases. Nonetheless, it may prove of some interest to take up in order these five possibilities in an attempt to determine how much of a rôle each of these factors may have played in the etiology of the group of macrocytic anemias under consideration in this paper.

A rather strong case can be made out for the view that the patients of this group were suffering from a macrocytic anemia as the result of deficient absorption of the hematopoietic principle such as is thought to occur in sprue. It seems quite possible that the enteritis set up by the intestional stagnation that so often occurs above strictures or in short-circuited loops of bowel may interfere with the absorption of this substance. This explanation, favored by a number of authors, must be given serious consideration at least in certain of the cases where absorption of glucose, fat, and the active principle of liver was shown to be impaired. Nevertheless, it could scarcely apply to all of the cases in this group, because in the majority of the cases in which intestinal absorption was studied, the results of the tests were surprisingly good.

As for the second possibility, unfortunately little was said in the case reports about the dietary of the patients of this group. In the two cases that we observed personally the anemia developed in spite of a normal intake of food and vitamins. It seems unlikely that deficiency of the extrinsic factor played more than a secondary etiologic rôle.

The occurrence of macrocytic anemia in association with disease of the liver is well established (Rosenberg, Wintrobe), but it is unusual to see a very marked degree of anemia under such circumstances. Although hepatic dysfunction undoubtedly was a factor in certain of the cases, there was little to suggest that this was of major importance. In our two cases conclusive evidence of liver damage was not found by any tests of liver function that were employed.

None of the etiologic mechanisms so far discussed furnishes a completely satisfactory explanation of the cases of macrocytic anemia under discussion. This leaves for consideration the last two possibilities: 4) failure of formation of the hematopoietic principle or destruction of the principle in the gastro-intestinal tract through abnormal bacterial activity, and 5) the absorption of toxic products of bacterial putrefaction in amounts too great to be neutralized by the normal detoxifying mechanisms of the body. These two possibilities will be discussed together because they rest upon a common basis, namely excessive bacterial activity in the intestinal tract.

Although the idea that the absorption of hemotoxic substances from the intestinal tract may be responsible for the development of "idiopathic" pernicious anemia is now an obsolescent theory, in the cases of macrocytic anemia associated with intestinal strictures and anastomoses such a theory must be given serious consideration. Hence it might be well to review briefly the evidence that has accumulated to support such a view.

When Faber in 1895 first called attention to the association of "pernicious anemia" with a stricture of the small intestine, he suggested that the anemia resulted from the absorption of a poison from the stagnant bowel contents. Meulengracht (1921) found at autopsy in a case of severe "pernicious anemia" associated with a tuberculous stricture of the ileum that the entire small intestine was heavily infected with bacteria. Comparing his case with five similar cases from the literature, he drew the following conclusions: 1) pernicious anemia may develop on the basis of benign intestinal strictures; 2) the anemia is probably due to the absorption of hemotoxic substances from the dilated and infected portion of the bowel above the stricture; 3) such cases support the theory of the intestinal origin of cryptogenetic pernicious anemia.

In 1927 Seyderhelm, Lehmann, and Wichels succeeded in simulating the clinical picture of stricture anemia in four of ten dogs in which they had produced intestinal strictures at operation. The anemia proved fatal in two of these dogs and post-mortem examination revealed marked stagnation and putrefaction of the bowel contents above the stricture with a luxuriant growth of bacteria throughout the small intestine. In the case of the dogs which failed to develop anemia the small intestine above the stricture remained practically sterile. The authors concluded that the absorption of toxic end-products of bacterial putrefaction was responsible for the development of the anemia. More recently Horster has added further experimental evidence along the same lines. He observed that dogs in which he had produced intestinal strictures or blind pouches developed an abundant flora of gram-negative bacilli in the upper intestinal tract and even in the stomach. Such dogs showed marked indicanuria, severe anemia with hemosiderin deposits in the liver and spleen, and evidence of liver damage. These changes could be prevented by feeding trichlorcresol to check the bacterial growth; furthermore the symptoms cleared up after resection of the blind pouch or stricture.

"Idiopathic" pernicious anemia was treated by means of ileostomy and colonic irrigations by Seyderhelm (1922) with interesting results. Complete blood remissions of five and seven months respectively occurred in two of three patients so treated, but relapse followed closure of the ileostomy. After ileostomy the drainage from the ileum became practically sterile in these two cases, whereas in the third case (a patient who derived no benefit from ileostomy) the drainage remained foul and grossly infected and at autopsy a heavy bacterial growth was noted throughout the small intestine. It was scarcely unreasonable for Seyderhelm to suppose that the improvement in the first two cases had resulted from the eradication of intestinal putrefaction.

Seyderhelm's observations were confirmed in 1925 by Dixon, Burns and Giffin in their report of six cases of pernicious anemia treated by ileostomy. Four of the six cases showed at least temporary blood remissions while symptomatic improvement was striking in several. These authors suggested that ileostomy, besides excluding the colon, presumably establishes more complete drainage of the ileum. Completely in accord with these observations are the bacteriologic studies of Otto and others who have described a luxuriant pathologic flora of colon bacilli throughout the entire gastro-intestinal tract of patients suffering from pernicious anemia. The above work represents only a small portion of the clinical and experimental data that has accumulated to make out a very strong case for the enterotoxic theory of pernicious anemia.

With the classic discovery of liver therapy for pernicious anemia by Minot and Murphy in 1926, soon to be followed by Castle's (1929) fundamental observations on the relation of the intrinsic (gastric) and extrinsic (food) factors to the hematopoietic principle of liver, the toxic theory gave way to the overwhelming evidence that pernicious anemia was a deficiency disease, or at least a "conditioned deficiency state." However, the mechanism of action of the liver principle is still far from clear. It has been postulated that liver supplies a substance necessary for the proper maturation of the erythrocytes. Such a substance must possess the additional properties of preventing or curing the other changes that occur in pernicious anemia such as the atrophy of the mucosa of the tongue and stomach and the degenerative lesions in the spinal cord. Furthermore, if we accept the view that pernicious anemia is a hemolytic anemia, a hypothesis which is strongly supported by the hyperbilirubinemia and the increased excretion of urobilin that occur regularly during the relapse phase of the disease, we must attribute still another property to the liver principle,—that of inhibiting hemolysis. It would then appear that this principle must exert its effect at several different sites in the body, yet it has already been demonstrated (Dakin and West) that a very small amount of the material is necessary to bring about improvement in all of the various manifestations of pernicious anemia. It is certainly tempting at least to assume that the liver principle might exert its effect at a single point: for example, in the liver itself or in the intestinal wall.

If the site of action were the liver, it seems possible that the anti-anemic principle may promote the detoxification of some injurious substance or substances absorbed from the intestinal tract. Detoxification of certain products of intestinal putrefaction such as indol and skatol has long been recognized as one of the important functions of the liver. The work of Rhoads and his co-workers indicates that a severe hemolytic anemia without reticulocytosis may be produced in dogs fed a deficient diet by the oral administration of indol and that this anemia may be prevented or cured by the oral administration of liver extract. Furthermore, Horster has shown that the various manifestations of intestinal intoxication (including anemia, liver damage, and hemosiderosis) which developed in his dogs with intestinal strictures or blind pouches could be inhibited or alleviated by injections of liver extract. Clinically macrocytic anemia commonly occurs in association with extensive disease of the liver. The usual explanation given for macrocytic anemia of this type is the failure of the damaged liver to store adequate amounts of the hematopoietic principle. However, it has been a common experience to find that in certain cases the macrocytic anemia of liver disease fails to respond well to therapy with liver extract. This observation suggests that the liver itself must be functioning well if the active principle of the injected extract is to work effectively. It does not require a wide departure from the established facts to look upon the manifestations of pernicious anemia as resulting from the penetration of some toxin beyond the normal liver barrier whence it might exert its effects at widely scattered points in the body.

On the other hand there is certain evidence that the liver principle may act directly in the intestinal wall. Various workers (Fairley; Hanes and McBride; Barker and Rhoads) have shown that the absorption of glucose and fat in cases of sprue is far better after treatment with liver extract than before such treatment. If, as it appears, the liver principle can increase the permeability of the intestinal wall for certain necessary food substances, it is quite conceivable that it may decrease the permeability of the wall to other substances, as for example toxic materials, or promote the detoxification of such materials in the intestinal wall.

The above discussion has been presented in the hope of reviving interest in the possibility that intestinal intoxication may be an important factor in the pathogenesis of pernicious anemia and related macrocytic anemias. We do not wish to create the impression that we are disputing the current view that a deficiency state is of primary importance in pernicious anemia. It must be pointed out, however, that the mode of action of the hematopoietic principle has not been explained. We are merely suggesting an explanation of the mechanism of action of liver therapy which is totally different from the generally accepted one. In other words, instead of regarding the active principle of liver as supplying a building-stone for the proper maturation of the red blood corpuscles, we feel that there is abundant evidence to support the contention that the liver principle may be necessary to promote the proper detoxification of some chemical compound or compounds, which if unneutralized might give rise to a variety of harmful changes throughout the body. This hypothesis is not an entirely novel one, for in addition to a number of European workers, Dock in this country has recently expressed himself as favoring such a stand. On the basis of such an hypothesis, the macrocytic anemias associated with intestinal strictures or anastomoses might be regarded as resulting from a state of abnormal intestinal toxemia which could be overcome either by removing the cause for the stagnation and putrefaction of the intestinal contents or by supplying excessive amounts of the liver principle to detoxify the excess of toxins absorbed. The development of true "idiopathic" pernicious anemia, on the other hand, would depend upon the failure of the body to synthesize the detoxifying principle in sufficient amounts to neutralize the toxic substances normally absorbed from an intestinal tract where stagnation is not necessarily a factor.

SUMMARY AND CONCLUSIONS

Two cases of macrocytic anemia in association with intestinal anastomoses have been reported in detail and 49 cases of a similar type of anemia occurring in patients with intestinal strictures or anastomoses have been reviewed from the literature.

From an analysis of these cases the conclusion seems justified that we are dealing with a distinct disease entity and not with a simple coincidence of "idiopathic" pernicious anemia and an intestinal lesion.

The pathogenesis of this form of macrocytic anemia appears to be intimately bound up with stagnation and putrefaction of the intestinal contents, resulting perhaps in the absorption of toxic substances. Consequently, successful treatment of the anemia usually depends upon the surgical removal of the cause for the intestinal stagnation, although intensive therapy with liver extract may bring about clinical and hematologic improvement in certain cases.

The relationship of macrocytic anemias of this type to "idiopathic" pernicious anemia has been discussed. It is suggested that the hematopoietic principle of liver may exert its effect, not by supplying a building-stone for the proper maturation of erythrocytes, but by promoting the detoxification of injurious substances absorbed from the intestinal tract.

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FIG. 3. *Case no. 49. Photomicrographs of the sternal bone marrow.* Mallory's eosin and methylene blue. (A) Before treatment. The marrow is hyperplastic with defective maturation of both erythroid and myeloid cells. × 250. (B) After treatment with diet for sprue and liver extract. Note the decrease in cellularity and the increase in fat. Maturation has returned to normal with normoblasts and polymorphonuclear leucocytes predominating. × 250.

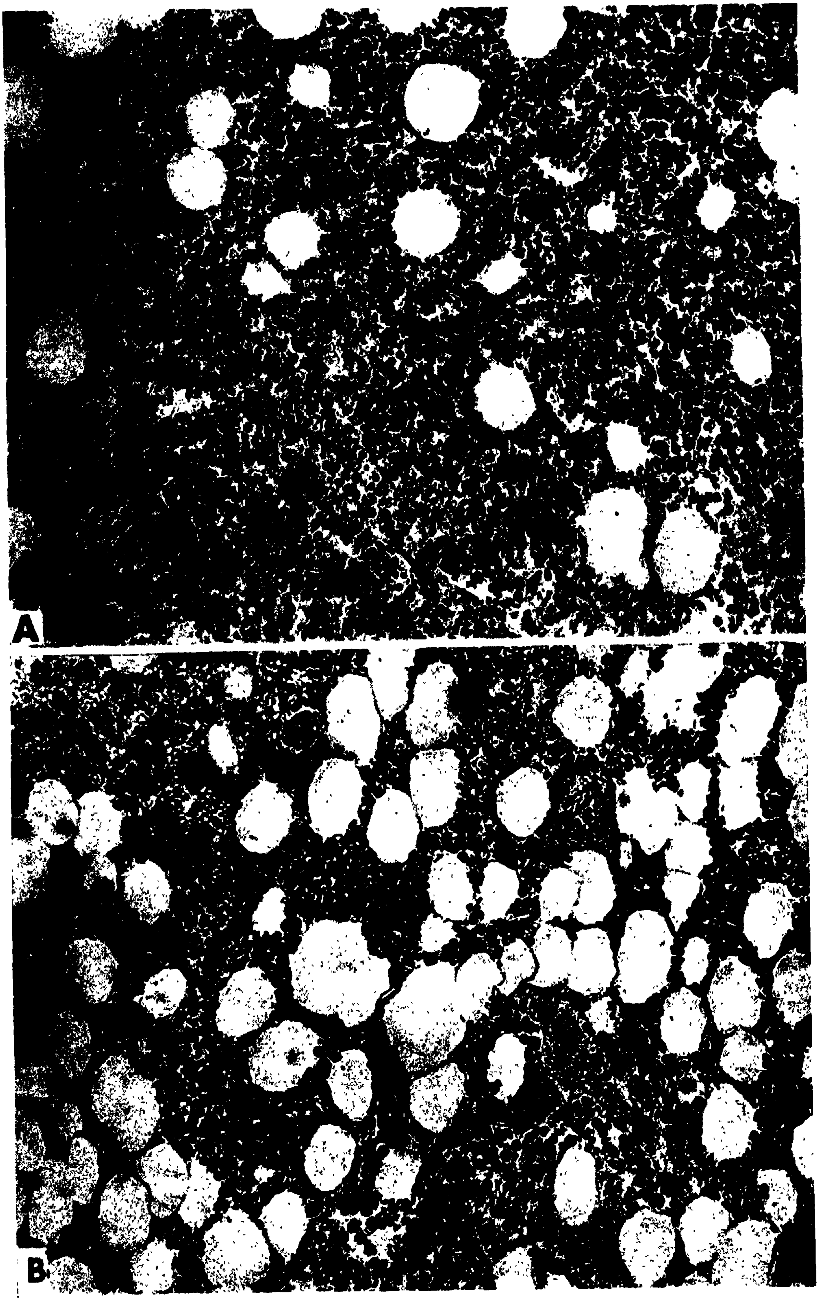

FIG. 3

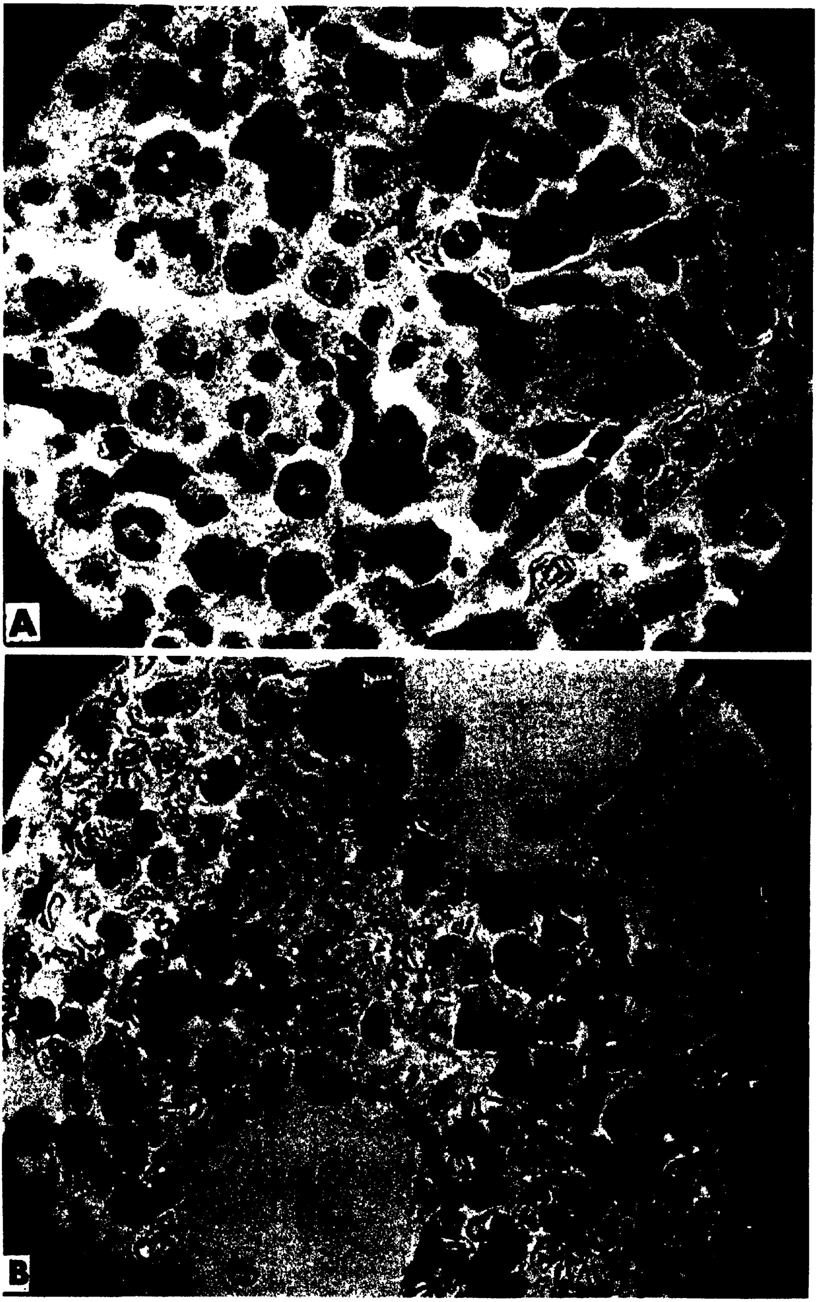

Fig. 4. (A) The same as Fig. 3 A. × 1000
(B) The same as Fig. 3 B. × 1000

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GROWTH OF THE FOWL CORYZA BODIES IN TISSUE CULTURE AND IN BLOOD AGAR

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The presence of small, Gram-negative, coccobacilliform bodies in nasal exudate from chickens infected with a coryza of slow onset and the cultivation of these bodies in a medium containing chick embryo tissue have already been reported (1, 2). In the present paper, observations on their nature and growth requirements, made during 3 years of nearly continuous cultivation, are brought together.

Five different strains of the coccobacilliform bodies (fowl coryza bodies) have been under cultivation for different periods during the past 3 years; 3 of these were isolated by filtration through large pored collodion membranes and 2 by direct inoculation of exudate. Unless otherwise stated the culture medium was that originally employed, consisting of approximately 75 mg. of finely minced 10 day chick embryo tissue suspended in 5 cc. of Seitz-filtered Tyrode solution in 15 mm. test tubes (2). The eyes were removed from the embryos prior to mincing. The volume of inoculum was usually 0.01 cc. Identification of the specific bodies was of necessity microscopic and was made from Gram-stained films using carbolfuchsin diluted 1:4 with distilled water as the counterstain. In our experience somewhat sharper staining was obtained when the entire Gram method was employed.

Growth Characteristics in Whole Tissue Culture Medium

The fowl coryza bodies were readily established and easily maintained in whole tissue culture media. The titer of the bodies in the supernatant of an actively growing culture, as determined by the subinoculation of serial dilutions, varied between 10^7 and 10^8 per cc. The results of a typical titration experiment are given in Table I.

In actively growing cultures, transferred at 48 hour intervals, the specific bodies were generally detectable microscopically after 1 day of incubation and regularly after 2 days. If the interval of transfer was prolonged, growth was usually delayed until the 3rd day and occasionally until the 4th. In 1 or 2 day cultures the bodies were demonstrable in both the supernatant and the sedimented tissue. In the supernatant they were generally few in number and discrete; in the tissue they were relatively numerous and often in large groupings, either within masses of disintegrating tissue or free. Occasionally they were present in intact tissue cells. After the 3rd day it was usually difficult to demonstrate them microscopically, due apparently to autolytic changes. There was no certain macroscopic evidence of growth in tissue culture media. In freshly prepared media inoculated with an actively growing culture there was often a faint turbidity at 24 hours which was not apparent in uninoculated but incubated tubes. This turbidity was readily obscured by autolytic changes and was not a reliable criterion of growth.

TABLE I

Titration of Fowl Coryza Bodies in Supernatant of a 48 Hour Tissue Culture

Dilution inoculated	Microscopic examination of culture
10^{-2}	+ after 24 hrs.
10^{-3}	+ " 24 "
10^{-4}	+ " 48 "
10^{-5}	+ " 48 "
10^{-6}	+ " 48 "
10^{-7}	+ " 72 "
10^{-8}*	– " 96 "
10^{-9}*	– " 96 "

* No growth in subculture inoculated with 0.1 cc. at 96 hours.

Growth in the Absence of Living Cells

The following group of experiments was carried out to determine whether living cells were essential for growth of the fowl coryza bodies.

Supernatant Cultures.—

Freshly prepared tissue culture medium was incubated at 37°C. for 48 hours and the slightly opalescent supernatant removed from the sedimented tissue, care being taken not to disturb the latter. The faint turbidity was due to autolytic products or precipitated salts and not to the presence of cells. The supernatants were inoculated and tested for growth, after 24 and 48 hours' incubation, by film examination and the subinoculation of serial dilutions.

Growth of the fowl coryza bodies was regularly supported by the supernatants of tissue culture media and also maintained on serial transfer. Microscopic examination showed sparsely distributed bodies, usually discrete but occasionally in small aggregates. The growth titer was practically identical with that of the supernatant of whole tissue culture medium, 5 titrations with 3 strains giving end-points of 10^7, 10^7, 10^8, 10^7, and 10^6 bodies per cc.

Heated Tissue Cultures.—Freshly prepared tissue cultures incubated at 37°C. for 24 hours and then heated to 60°, 70°, 80°, 90°, and 100°C., respectively, for 1 hour in a water bath supported an active multiplication of the fowl coryza bodies. In comparison with unheated media there was usually a reduction in the number of bodies within tissue fragments, a marked fixation of the tissue being apparent at the higher temperatures. The supernatants, however, tested by subinoculation, showed a growth titer comparable to that of unheated cultures, growth being obtained through a dilution of 10^{-7} in 3 lots of media heated for 1 hour at 100°C. The appearance of the bodies in heated cultures was often delayed until the 3rd day. Growth was also maintained in serial transfers, one strain being carried through 10 successive culture passages. There was no detectable growth either microscopically or by subinoculation in tissue cultures heated in the autoclave for 15 minutes at 15 pounds pressure.

Aged Tissue Cultures.—Tissue cultures which had been incubated for 48 hours at 37°C. and then held at 10°C. for periods of 2 to 8 weeks regularly supported growth of the fowl coryza bodies, though microscopic examination indicated that growth in the tissue fragments was less than in freshly prepared media. The supernatants, however, regularly gave an end-point of 10^{-7} on subcultivation in dilutions. Growth was also maintained in media held for longer periods. In such tubes there was considerable evaporation of fluid and the original volume was restored by adding sterile water. The supernatant of one lot of medium stored for 6 months gave a growth titer of 10^6.

Growth in the Sediment and Supernatant Fractions of Stored Media

The preceding observations indicated that living cells were not essential for multiplication of the fowl coryza bodies and suggested that growth was conditioned by some diffusible component of the tissues. Further evidence was afforded by the following experiments in which the tissue sediments and the supernatants of media were tested separately at different time intervals.

Freshly prepared tissue culture media were incubated at 37°C. for 48 hours and then stored at 10°C. At the end of each week for 5 weeks the supernatants were transferred to sterile tubes and 5 cc. of normal Tyrode solution added to the nearly fluid free sediments. After inoculation the respective cultures were examined microscopically through the 4th day. If the specific bodies were not demonstrated in films or if their growth was less than normal the supernatants were subcultured in dilution, usually on the 3rd day.

The results of this experiment are summarized in Table II. At the end of the 1st week of storage both tissue culture fractions supported a normal growth of the fowl coryza bodies. At the end of the 2nd week there was an indication of reduced growth in the sediment fraction. At the end of the 3rd week there was a significant reduction of growth in the sediment, the titer on subculture being 10^2. Growth in the supernatant, however, was unaffected. After 4 weeks of storage there was no demonstrable growth in the sediment, although the growth-promoting capacity of the supernatant remained unchanged. After 5 weeks of storage there was still a scant growth in the sediment fraction, the titer on subculture being 10^1. The supernatant continued to support a normal growth, with a titer of 10^8 bodies per cc. This experiment was repeated with a second lot of tissue culture media with essentially the same results, indicative of a complete or nearly complete exhaustion of the tissue on storage.

TABLE II

Growth of Fowl Coryza Bodies in Supernatant and Sediment Fractions of Stored Media

Period of storage	Growth in supernatant		Growth in sediment	
	Microscopical examination	Titer on subinoculation	Microscopical examination	Titer on subinoculation
1 wk.	+ Normal		+ Normal	
2 wks.	+ "		+ Reduced	10^5
3 "	+ "	10^7	−	10^2
4 "	+ "	10^7	−	−
5 "	+ "	10^8	−	10^1

Growth in the Diluted Supernatants of Tissue Culture Media

On the basis of the preceding evidence it seemed permissible to refer the growth-promoting capacity of embryonic chick tissue to a diffusible cellular component. Dilution tests on the supernatants of tissue culture media, prior to and after incubation, were subsequently made to determine the concentration of this component.

The supernatants were removed from tissue culture media immediately after preparation, after 48 hours of incubation at 37°C., and after 5 days of storage at 10°C. following incubation. They were tested undiluted and in dilutions of 1:2.5, 1:5, and 1:10. The total volume of fluid was 5 cc. in each tube, the diluent being Tyrode solution. As a control, 5 cc. of normal Tyrode solution was tested with the same inoculum. The tubes were incubated at 37°C., examined microscopically through the 3rd day, and subcultures, using 0.1 and 0.01 cc. of inoculum, were made at intervals.

The undiluted supernatant of freshly prepared tissue culture media supported a sparse growth of the fowl coryza bodies, detectable only by subculture. There was no growth in the 1:2.5 or 1:5 dilution. The supernatant removed after 48 hours' incubation supported a normal growth, as indicated by microscopic examination, in undiluted state, and in the 1:2.5 dilution. There was a sparse growth in the 1:5 dilution, detectable only in subculture. The supernatant stored at 10°C. for 5 days also supported growth through the 1:5 dilution. The 1:10 dilutions were negative throughout. There was neither growth nor survival of the coryza bodies in normal Tyrode solution, 10^{-1} subcultures being regularly negative.

Growth in Tissue Culture Media Prepared with Sodium Bicarbonate-Free Tyrode Solution

The Tyrode solution heretofore employed contained 1 gm. of $NaHCO_3$ per liter of water. A number of experiments were made to determine the growth capacity of tissue culture media in which the $NaHCO_3$ was reduced or absent. Multiplication of the fowl coryza bodies was not significantly affected by half the normal amount (0.05 per cent). There was complete inhibition of growth, however, in the absence of $NaHCO_3$. Three different strains were tested and in no case were the specific bodies detectable either by microscopic examination or subcultivation.

The reaction of uninoculated tissue culture media prepared with $NaHCO_3$-free Tyrode solution and incubated for 48 hours at 37°C. was pH 6,[1] or lower, whereas the pH in the presence of the normal amount of $NaHCO_3$ under similar conditions was 7.3. The reactions of the respective media prior to incubation were 6.8 and 7.6. In the absence of $NaHCO_3$ there was little or no neutralization of the acidic substances produced either as waste products of the tissue or by autolysis. Both the fowl coryza bodies and the growth-promoting component were apparently affected by the low pH in the absence of $NaHCO_3$. In the case of the former, replacement of the normal supernatant of an actively growing 24 hour tissue culture by $NaHCO_3$-free Tyrode solution resulted in partial sterilization of the tissue fraction after 24 hours' further incubation and in complete sterilization after 48 hours. Concerning the latter, there was no detectable multiplication of the fowl coryza bodies in tissue culture media on replacement of the $NaHCO_3$-free supernatant by normal Tyrode solution after incubation at 37°C. for 48 hours.

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[1] The pH determinations were made colorimetrically by the writer and were checked potentiometrically in the glass electrode through the courtesy of Dr. Max Lauffer.

Viability of the Fowl Coryza Bodies in Tissue Culture Media

In media prepared with normal Tyrode solution the fowl coryza bodies remained viable for several weeks, though generally not demonstrable microscopically after the 3rd day. A delayed growth was regularly obtained with 0.1 cc. of an inoculum of cultures stored at 10°C. for 2 weeks. After 3 weeks of storage irregular results were obtained and after 4 weeks there was no detectable growth.

Preliminary observations on desiccation by the Flosdorf-Mudd method suggested that preservation might be conditioned by the age of the culture, in respect to number of generations. One strain which had been subcultured 112 times prior to desiccation was viable and infective 12 months thereafter. A second strain transferred 26 times was not cultivable on the 4th month after desiccation.

Growth in Tissue Culture Media Prepared from Postembryonic Organs

Tissue culture media were made with minced spleen and liver from 10 to 12 week old chickens to determine whether postembryonic tissue was suitable for growth of the fowl coryza bodies. A complicating factor in the use of these media was the frequency of bacterial contamination which had been negligible with chick embryo tissue. In addition, stained films usually contained so many miscellaneous granules that microscopic identification of the specific bodies was uncertain. They were, however, occasionally demonstrable by microscopic examination in spleen cultures but not in liver cultures. It was hence generally necessary to rely on subcultivation for the evaluation of growth, with highly variable findings. The results with spleen media inoculated immediately after preparation varied from no growth on subculture to normal growth with a titer of 10^7. In liver media no growth was often obtained, and in the presence of growth the titer was low, not exceeding 10^3. Media stored at 10°C. for 5 days after being incubated at 37°C. for 48 hours usually failed to support growth of the fowl coryza bodies even though they were demonstrable in other tubes of the same lot of medium inoculated immediately after preparation. The outcome of a typical growth experiment using spleen and liver media is indicated in Table III.

TABLE III

Growth of Fowl Coryza Bodies in Spleen and Liver Cultures

Time of inoculation	Titration of growth in spleen culture	Titration of growth in liver culture
Immediately after preparation	$10^6 + 72$ hrs.	$10^2 + 72$ hrs.
	$10^7 + 72$ "	$10^3 + 72$ "
	$10^8 + 96$ "	$10^4 - 96$ "
After storage for 7 days	$10^1 - 96$ "	$10^1 - 96$ "
	$10^2 - 96$ "	$10^2 - 96$ "

Growth in Nutrient Media Enriched with Blood

In the early work on the coryza of slow onset many attempts were made to cultivate the specific bodies in nutrient media enriched with horse or chicken blood. At that time no evidence of growth was obtained either in fluid blood broth mixtures or on solid blood agar. Later, with the introduction of tissue cultures as a means of checking growth cultivation was again attempted in blood media.

Slanted veal infusion agar (pH 7.6) containing 1 cc. of fluid defibrinated horse or chicken blood at the base of the slant was regularly employed. The initial inoculum unless otherwise stated was 0.01 cc. of a 24 or 48 hour tissue culture. From the primary culture 5 or more serial transfers were made at intervals of 3 days, using the same volume of inoculum. With each transfer a tube of tissue culture medium was also inoculated. Both cultures were incubated at 37°C. and examined microscopically through the 4th day.

Five recently isolated strains of the fowl coryza bodies were tested in horse blood agar and 2 in chicken blood agar. There was no microscopic evidence of growth in any of the primary blood agar cultures or in the subsequent transfers. Tissue cultures inoculated from the 1st and in some cases the 2nd blood agar tubes showed a delayed growth. Coccobacilliform bodies were not demonstrable, however, in tissue subcultures from the later blood agar transfers. These results are interpreted as indicating merely a survival of the specific bodies introduced with the initial inoculum and not indicative of actual multiplication.

From time to time additional series of transfers to horse blood agar slants were made from later tissue culture generations. With continued tissue culture propagation a significant change in the growth requirements of one strain was observed. Through the 60th tissue culture generation of this strain there was no evidence of growth in blood agar subcultures. Beginning with the 80th generation there was an indication of longer survival but the results were irregular. With the 120th generation, however, growth was definitely established and maintained through 125 biweekly transfers. From the 4th blood agar culture on, the specific bodies were directly detectable in Gram-stained films of the fluid mixture at the base of the slant.

Sharply stained and defined bodies were observed as early as 24 hours after incubation at 37°C. In the early transfers they were few in number and usually discrete or paired. In later transfers the bodies were more readily detectable but never numerous. There was also a greater tendency to form aggregates some of which were large and compact resembling the colony-like clump characteristic of the X bacillus (1). Horse blood agar cultures showed no macroscopic evidence of growth, nor was colony formation observed on horse or chicken blood agar plates containing up to 20 per cent blood.

Coincident with the above noted cultural changes in the fowl coryza bodies on continued cultivation in blood agar was an alteration in virulence. Through the 27th generation the specific bodies produced a characteristic coryza on nasal injection in normal fowl. The 83rd generation, however, was avirulent and failed to initiate any demonstrable reaction in the host. The parent culture of this strain maintained by continued tissue culture transfer retained its infectivity through many subsequent generations.

The adapted strain of the coccobacilliform bodies was finally lost by contamination and a second strain was established in horse blood agar from the 190th tissue culture generation. This strain, now in its 100th transfer, has behaved in much the same way as the first. It is now avirulent and shows aggregate formation in films.

DISCUSSION

The preceding growth tests on the coccobacilliform bodies of fowl coryza indicate that they can be maintained for an indefinite number of generations in tissue cultures, the limit of their viability in each being 2 to 3 weeks. Artificial cultivation has no immediate effect on their infectivity. A sufficient number of strains has not been tested to warrant a statement on the effect of prolonged cultivation; one strain, however, was pathogenic after more than 200 subcultures.

It is evident that the capacity of embryonic tissue to support growth of the fowl coryza bodies is not dependent on the presence of living cells, as such. Multiplication is regularly demonstrable in supernatants on removal from the sedimented tissue, in media heated to a sufficiently high temperature to kill all of the tissue cells, and in media in which the cells are inactivated by prolonged storage. This fact is valid evidence of the presence of a specific component of the tissue which is essential for growth of these bodies.

On incubation of the tissue culture medium, this cellular component or growth factor passes from the sedimented tissue to the supernatant in a sufficient amount to promote multiplication of the fowl coryza bodies. On storage for 4 weeks or longer, the growth capacity of the supernatant is retained undiminished, whereas that of the tissue is exhausted or nearly so. It is probable that this depletion is largely due to diffusion of the growth factor from the tissue to the surrounding fluid, but other factors, such as inactivation by autolysis, must also be considered. It may be noted that the tissue after depletion of the essential growth factor still contains enough nutritive material to promote a fair multiplication of ordinary bacteria. The concentration of this growth factor in tissue culture supernatants, as indicated by dilution tests, is low, 1:5 being the highest dilution in which multiplication occurs. The supernatant of tissue culture media on removal immediately after preparation may support a feeble growth of the specific bodies, indicating that a trace of the essential factor is either washed from the tissue or liberated by injury to the cells.

A limited and variable amount of growth factor which tends to deteriorate rapidly on storage is demonstrable in the spleen and liver of 10 to 12 week old chickens. These organs are not a satisfactory substitute, however, for embryonic tissue. Nutrient media enriched with horse or chicken blood do not contain a sufficient amount of the essential factor to support growth of recently isolated strains of the fowl coryza bodies. The adaptation of one strain to horse blood agar after many tissue culture generations may signify that a trace of this factor is present. It may mean, however, that continued cultivation brings about a qualitative change in the nutritive demands of the specific bodies whereby they are able to multiply in the absence of the growth component.

The chemical nature of the assumed essential growth factor is unknown. The effects of environmental factors on its activity include heat stability at 100°C., stability on prolonged storage in tissue culture supernatants, and sensitivity to pH values of 6.0 or lower.

Although the fowl coryza bodies bear a superficial morphological resemblance to the elementary bodies and the rickettsiae, they appear to be separable from the latter on physiological grounds. According to our present knowledge multiplication of the elementary bodies is strictly intracellular and conditioned by the presence of living tissue cells. Intracellular development is likewise postulated for the rickettsiae. The recent observations of Zinsser and Schoenbach (3) indicate, however, that the cellular requirements of the rickettsiae are quite different from those of the viruses, multiplication being favored by metabolically inactive tissue cells. It is of particular significance, in connection with the fowl coryza bodies, that the growth capacity of embryonic tissue for typhus fever rickettsiae is destroyed at a temperature of 50°C. In apparent contrast to the multiplication of the elementary bodies and the rickettsiae that of the fowl coryza bodies is essentially extracellular, but it should be borne in mind that a cell-restricted development of the former is still an assumption and not a fact.

That the fowl coryza bodies are living and properly classified with the bacteria is strongly suggested by their extracellular manner of growth, and in particular by the adaptation of one strain to blood agar. They are closely related to the infective agent of mouse catarrh (4), but they appear to have no counterpart among the known bacteria.

In size they are, although somewhat larger, comparable to the granular forms of the much discussed agent of bovine pleuropneumonia, for which Turner (5) has suggested the generic name Borrelomyces, and to the sewage organisms of Laidlaw and Elford (6) which according to Ørskov (7) are morphologically identical. Up to the present time the fowl coryza bodies have failed to exhibit the complex morphology which characterizes the organisms of the pleuropneumonia group. Since it is possible that similar forms may be demonstrable by other technical procedures in the hands of other investigators, we prefer at present to retain the descriptive name of fowl coryza or coccobacilliform body in lieu of establishing a new bacterial genus.

SUMMARY

Evidence is presented that the growth capacity of chick embryo tissue for the fowl coryza bodies is conditioned by a diffusible cellular component which is essential for their multiplication. This growth factor is inactivated at pH 6, but withstands a temperature of 100°C. for 60 minutes. An amount sufficient to promote a normal growth of the specific bodies may be present in tissue culture supernatants long after its content in the tissue is exhausted.

Postembryonic tissue (liver and spleen) contains a variable amount of growth factor and is not a satisfactory substitute for the chick embryo. Multiplication of recently isolated fowl coryza bodies is not demonstrable in nutrient media enriched with blood. Experiments with one strain, however, indicate that an adaptation to fluid blood in an agar medium may take place after many generations in tissue culture.

The probable bacterial nature of the fowl coryza bodies is discussed on the basis of their cultural requirements.

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HEREDITARY BRACHYDACTYLIA AND ALLIED ABNORMALITIES IN THE RABBIT

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PLATES 15 TO 17

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The investigation of brachydactylia and related deformities in man has been limited in large part to a genetic study. The frequent recurrence of malformations in succeeding generations of a family led to an early recognition of their hereditary nature, and the scheme of inheritance of brachydactylia was accurately described forty-three years before the rediscovery of Mendel's laws (1). Other types of deformity involving an absence or shortening of the parts of the hands and feet have since been shown to follow the general laws of genetics, but the investigation of more detailed genetic problems has been limited by the lack of suitable data.

Anatomical studies based on x-ray examination and postmortem dissection have shown that the deformities arise from a partial or complete absence of the component bones. The deformities thus represent a gross developmental fault and are of pathological as well as of genetic interest. Their pathogenesis has not been studied in man, but the conditions observed by Streeter (2) in human feti and classified as intrauterine amputation suggest a final stage in their development.

In view of the nature of the material and the impracticability of controlled breeding experiments, progress in the investigation of human deformities remains dependent upon fortuitous circumstances. The occurrence of comparable abnormalities in lower animals, however, offers an experimental approach to the problems. Hereditary deformities of this order have been reported in the mouse (3) and in the rabbit (4). The object of the present paper is to describe the deformities as they occur in the rabbit and to report experiments concerned with their inheritance and pathogenesis.

Materials and Methods

The first brachydactylous deformity was discovered in 1932 in the offspring of apparently normal animals. The parents were litter mates derived from the crossing of a pure bred English doe with a hybrid male. Both parental lines had been bred for generations, and there were instances in which matings had been made which should have disclosed the presence of the deformity in either line, but none occurred until the cross indicated above was made.

The abnormality was limited to the feet in the first deformed animal and in all its immediate progeny. The ear defects did not occur until after continued breeding of the line, during the course of which a number of outcrosses were made with animals of different breed.

The various types of deformity were studied by dissection and x-ray examination at different age periods, and an investigation of the pathogenesis of the disorder was carried out on feti obtained at different stages of development. Tissues for microscopic examination were fixed in Petrunkevitch's solution and stained with hematoxylin and eosin.

F_1, F_2 and backcross generations were obtained from affected animals for hereditary studies. Investigation had shown that the abnormality was completely expressed at birth, and statistical studies were based on observations made at that time.

Types of Deformity

The deformities under consideration are characterized by a shortening or absence of the component parts of the feet. Extreme variations in the degree of malformation occur, giving rise to a series of abnormalities ranging from minor brachydactylia to complete acheiropodia.

One or all of the feet of an animal may be affected. As a rule, there is no apparent tendency toward lateral or horizontal symmetry either as regards the site or extent of abnormality.

The irregular involvement of different portions of the foot gives rise to a complicated diversity of clinical types, but three general grades of malformation may be recognized. The major abnormality is confined to the digits in one grade (Fig. 1); in another the metatarsal area is affected, while in a third the alteration extends into the region of the wrist and foreleg (Fig. 2). It should be emphasized, however, that these grades are not clear cut, and in the majority of instances some degree of abnormality is present in contiguous regions.

The reduction in length in digital deformities may be so extreme that only truncated stubs remain, or so slight that detection depends on comparative measurements. Dissection and x-ray examination show that deformities of this type arise from a shortening or an absence of phalanges. Shortened phalanges may be otherwise unaltered, but generally assume a constricted hour-glass shape. In many instances the bones are reduced to mere spicules, and frequently no indication of their presence remains (Figs. 3 and 4).

Multiple abnormalities may be present in a single digit, but occasionally only one phalanx is affected. In such cases the middle phalanx is most frequently involved, and the abnormality in this element is more often manifest by complete absence than by reduction in length. In other instances, the alteration of phalanges is extremely irregular, and both shortening and absence are observed in different situations in the same digit.

Usually more than one digit is affected. All of the toes of a foot may be involved to an equal degree, or adjacent digits in one portion may be absent or markedly deformed while those of another part are normal in appearance or only slightly altered. Abnormal phalanges are often found in corresponding positions in adjacent digits, but the degree of abnormality may vary greatly in the analogous bones.

As a rule, the first or most medially placed digit is least affected. This is particularly noticeable in the marked grades of digital shortening in which the remnants of affected digits are not separated from one another, but form a club-shaped stump from which the relatively normal thumb protrudes as a free and distinct unit.

Minor changes in the metatarsals, such as alterations in contour and decrease in diameter or in length, may accompany malformation of the corresponding phalanges, but usually marked abnormality of these bones is only found associated with complete absence of the digits. In extensive deformities the metatarsals may be absent, but marked shortening with a reduction to short pointed spicules is more commonly found (Figs. 5, 6 and 7).

Abnormalities in the tarsus and carpus, as in other parts of the foot, may be expressed in absence or in reduction in the size of individual elements. The most pronounced alterations occur in grades of deformity in which the metatarsals are absent (Fig. 8). The distribution of abnormalities is extremely irregular, but in general the most marked malformations are found in localized areas, while bones in other positions appear normal in structure or only slightly altered.

A decrease in the diameter of the distal extremities of the long bones of the foreleg often occurs jointly with deformities of this order, while an irregular reduction in the length of the bones which may involve the greater part of their shafts is sometimes associated with complete absence of the foot. In other animals, absence of the foot is accompanied by a uniform reduction in the diameter and length of the long bones which are otherwise without gross defect. X-ray examination, however, shows a marked thinning of the cortex in such instances.

Abnormalities of the nails may accompany any grade of deformity but occur with the greatest frequency in the more extensive types. The nails may be absent, distorted, or rudimentary in form, and in the latter conditions are not attached to the underlying bone. Alterations of the soft parts of the foot conform to the bony involvement.

Associated Deformities of the Ear.—Abnormalities of the ear may occur in association with malformations of the feet and, like these deformities, are characterized by the absence of a constituent part (Figs. 1 and 9). In contrast to the foot deformities, however, the defect in the ear occurs in a sharply localized area rather than in multiple foci, and other portions show no alteration. Moreover, the defects always involve the free border and are never limited to an internal part.

The extent of the abnormality is variable. In some instances the entire distal third of the ear is missing, while in others only a blunted appearance due to absence of the pointed tip distinguishes the ear from normal. Occasionally, the extremity of the ear is not involved and the deformity is limited to the lateral border. Defects of the medial border have not been observed. One or both ears may be involved, and in the latter case the malformations are rarely symmetrical.

The defects are clear cut with regular smooth borders and may involve both fleshy parts and cartilage. The skin bounding a defective area is not altered, normal hair is present, and the general appearance is in direct contrast to that noted in cases where portions of the ear have been amputated in early extrauterine life.

Numerous animals of this line have been under observation, but other structural abnormalities have not been found with any degree of regularity. Opacities of the lens and cryptorchism occur in some instances, but the incidence is no greater than is found in the remainder of the colony. Particular attention has been directed toward examination of internal organs, and with a single exception no gross variations have been noted. The exception occurred in the liver of an adult animal which presented a gross structural abnormality with wide defects in its substance and an anomalous lobular division suggesting a developmental origin similar to that of the foot and ear deformities.

The deformities do not interfere with ordinary cage life, and affected animals lead a normal healthy existence. The early history of the line was characterized by a low fertility rate, reduced litter size, and poor maternal care. Such deficiencies were so pronounced in early breeding experiments that, despite continued efforts, two years elapsed before a sufficient number of animals could be raised to allow for genetic study. Affected males were extremely pugnacious and usually infertile while females rarely attempted to care for their young and fostering was necessary in nearly all cases. On the other hand, in later experiments, maternal care improved and fertility and litter size increased to the normal expectation.

Inheritance

The deformities described above occur in a single genetic line of rabbits. Similar malformations are rarely found in the general animal population, and in such instances, the abnormalities are apparently determined by different factors, for they do not recur in subsequent inbred generations or in litters obtained by crossing with the brachydactylous line.

In the brachydactylous line, on the other hand, the deformities "breed true," and all animals derived from deformed parents are affected. The site and extent of abnormality, however, may vary greatly in parents and progeny. For this reason statistical studies in the present report are based on affected individuals rather than on the recurrence of a specific type of deformity.

First generation progeny were obtained from animals with widely dissimilar types of foot deformity and all showed normal feet. In one group of experiments the animals were interbred and backcrossed without regard to the site or form of the parental abnormality. For example, F_1 animals derived from a parent with minor brachydactylia of all feet were backcrossed to animals in which the deformity was limited to acheiropodia of a single leg or *vice versa*, while F_2 generations were produced by various types of line crosses. In another group of experiments, breeding was confined to sister-brother or parent-progeny matings.

The proportion of affected progeny in the resulting generations was the same whether the F_1's were mated within their own families, or were crossed with animals representing different forms and locations of abnormality. All of the various types of malformation occurred in these generations, but in no instance was the parental deformity exactly duplicated.

The ratio of animals with normal and deformed feet in F_2 and in backcross generations is shown in Table I. The ratios approximate those expected in the inheritance of a simple recessive character and the differences are not statistically significant. Both males and females were found to transmit the variation, and the proportion of affected males and females was approximately equal in the different generations. The condition is, therefore, neither linked to sex in transmission nor influenced by sex in expression, but is dependent upon a simple recessive unit factor.

TABLE I

The Ratio of Rabbits with Normal and Abnormal Feet in F_2 and Backcross Generations

Generation	Normal feet		Abnormal feet	
	♂	♀	♂	♀
F_2	101	95	31	39
Actual total	196		70	
Expected total	199.5		66.5	
Backcross				
F_1 ♀ × abnormal ♂	71	53	51	48
F_1 ♂ × abnormal ♀	86	66	103	89
	157	119	154	137
Actual total	276		291	
Expected total	283.5		283.5	

TABLE II

The Ratio of Rabbits with Normal and Abnormal Ears in Backcross Generations

	Normal ears	Abnormal ears
Normal feet	116	0
Abnormal feet	95	27
Actual total	211	27
Expected total	119	119

The ratio of normal and abnormal progeny in backcross generations obtained from animals with ear defects is shown in Table II. It was found that all F_1 animals derived from affected males and females possessed normal ears but transmitted the abnormal condition. Abnormal ears recurred in only 11.3 per cent of the backcross progeny rather than in 50 per cent, as would be expected in the inheritance of an independent recessive character. Moreover, the occurrence of defective ears was limited to animals with deformed feet. The ratio of progeny with abnormal feet conformed to that observed in previous experiments. In addition, 22.1 per cent showed defective ears.

Statistical analysis shows that the association of abnormal ears with deformed feet is not a matter of chance. The factors concerned in the determination of the two abnormalities are, therefore, either closely linked or identical. Despite numerous additional breeding tests, the ear abnormality has never been obtained as a separate entity, and it seems improbable that the association is due to linkage. On the other hand, evidence obtained from more detailed genetic studies to be reported in a later paper indicates that the two deformities arise from the same fundamental variation. This evidence is supported by morphological studies described in the following paragraphs, which show that the pathogenesis of the abnormality is identical in both locations.

Embryological Examination

During the latter half of gestation, feti derived from the interbreeding of affected animals show pathological changes at sites corresponding to the deformities observed at birth. The nature and extent of the alterations are comparable in unrelated feti of the same age, and a sequence of changes beginning with the first appearance of abnormality and terminating in the complete deformity may be observed in a series of animals obtained between the 16th and 25th days of gestation.

Gross Examination.—Gross abnormal changes were not found before the 18th day of gestation despite the careful examination of a great many younger feti. The abnormalities noted at this time were situated near the surface of the developing foot and ear buds and consisted of areas of red discoloration (Fig. 10). The discolorations varied in size from small punctate spots to areas involving the greater part of the bud and were usually slightly elevated. Small lesions deep in the tissues were found on histological examination but were not observed in the gross.

On the 19th day the involved areas were generally more sharply outlined and were of a deep red color (Fig. 11). The larger discolored regions were swollen, but the discoloration and swelling stopped abruptly at the junction with normal appearing tissue. The smaller lesions often appeared as minute red cysts which exuded bloody material when pricked but ceased bleeding even in living animals as soon as the immediate area was drained. Small cysts were frequently found in different parts of the same foot but were never observed in multiple positions in the ear.

A line of demarcation similar to that observed in gangrenous lesions was apparent on the 20th or 21st day separating abnormal and normal parts (Figs. 12 and 13). An annular constriction subsequently appeared in this region, and by the 23rd day the affected portion had sloughed off or remained as a shrunken necrotic area. Sloughing had occurred and the stub had apparently healed in all cases examined after the 25th day (Fig. 14). The process did not recur in the subsequent days of gestation, and feti examined after the 25th day showed a deformity comparable to that noted at birth.

Histological Examination.—Identical histological changes were observed in the developing foot and ear buds, but tissues in other situations showed no alteration from normal. Sections from feti killed on the 16th and 17th days of gestation showed no other abnormality than a dilatation of thin walled vessels with occasional small areas of extravasated blood. Degenerative changes were not observed. Blood vessels and nerves were normally distributed throughout the bud and were intact and unaltered in structure.

Numerous areas of hemorrhage in the muscle and subcutaneous tissue were present in 18 day old feti. Small hemorrhages were not accompanied by other change, but the larger extravasations were associated with necrosis of the areas involved.

Necrosis dominated the picture from the 19th day to the 21st day (Figs. 15 and 16). In many instances the greater part of a member was involved in the process, while in others necrosis was limited to a small area. The large necrotic areas in the foot frequently extended from the tarsal or carpal bones to the digital extremities, and all intervening tissues showed complete dissolution of cell structure with pycnosis and karyorrhexis of nuclei. Smaller necrotic foci were often multiple and scattered irregularly in internal and peripheral areas. They occurred both in widely separated regions and in closely approximating positions. In the latter case, the lesions remained discrete throughout and did not coalesce by extension.

The large areas of necrosis frequently involved all the digits of a foot with the exception of the first metatarsal and its phalanges, but otherwise the distribution of lesions followed no recognizable order. Different regions were affected with equal frequency and there was no apparent site of predilection either in regard to different tissues or to specific locations in these tissues. Foci of necrosis occurred with the same incidence in the extremities and shafts of developing bones, and at the center and periphery of muscle anlage. The lesions were seldom limited to any one tissue, but involved parts of cartilage, muscle, vessels, and nerves.

All tissues and regions of the developing buds with the exception of the epidermal epithelium were affected in different animals. This tissue remained intact and unaltered in structure despite the involvement of all other elements or the presence of a necrotic lesion in the immediate underlying area.

Necrosis was always associated with extravasations of blood. The larger lesions were irregularly and loosely infiltrated with blood cells, but in the smaller foci, the cells were often arranged in compact masses resembling hematomata (Fig. 17). Hemolysis, however, did not occur, and the cells were always intact and well preserved in contrast to the surrounding necrotic tissue. Nucleated red cells were present in greater concentration than in the normal blood vessels of the part, suggesting that the extravasation was of some duration at the time of examination and that the preservation of the blood cells in the necrotic area was related to some special property.

The necrotic areas were usually irregular and asymmetrical in shape, and with the exception of blood cysts close to the surface, sharply circumscribed lesions did not occur. In general the tissue in the region of heaviest blood extravasation was uniformly involved, but the process extended irregularly into the surrounding tissue, and the position of maximum hemorrhage was not constant in relation to the area of necrosis.

The process of healing was apparent in many instances as early as the 20th day and was usually completed by the 25th day. The necrotic material in internal lesions was removed by phagocytosis and the defect partially filled by a loose proliferation of fibroblasts. The removal of large necrotic areas and small superficial lesions, on the other hand, was executed by sloughing. Sequestration of the affected region was accomplished by a downgrowth of epithelium from normal parts which began between the 20th and 21st days and completely separated living and necrotic tissue before sloughing occurred (Fig. 18). The preservation of intact, normal appearing epithelium about the sequestrum despite the absence of any blood supply was a notable feature in the majority of cases.

DISCUSSION

Brachydactylia was the first physical variation shown to follow the laws of Mendelian inheritance in man. It is of interest that the same variation was the first hereditary deformity observed in a large breeding colony of rabbits and that, despite the subsequent discovery of numerous other genetic abnormalities, it remains at the present time the only one found to occur in accordance with simple Mendelian laws.

The recent use of the x-ray in the study of brachydactylous deformities in man has demonstrated the great variability in structural detail exhibited by the affected members of a given family. The conformity of anatomical changes described in the older literature was apparently based on limited examination and cannot be held as opposing an analogy with the deformities described above. On the other hand, many points of similarity suggest the existence of such a relationship.

The deformities in man are inherited in the manner of dominant Mendelian characteristics, while the variations in the rabbit are determined by recessive factors. This difference, however, is commonly observed in a comparison of the inheritance of the same abnormality in man and in lower animals.

The deformities in man vary from slight digital shortening to the complete absence of hands and feet in different families, but intermarriage has not been recorded and it has not been determined whether the various types arise from different genetic changes or represent modified forms of the same fundamental variation. Experimental breeding of rabbits, however, has shown that the different types of deformity are not distinct hereditary entities but form a genetically related series.

The presence of some degree of abnormality in one or other of the feet is regularly transmitted from parents to progeny, but the distribution and extent of the malformations are subject to extreme variation in succeeding generations. Genetic investigation is therefore concerned with two problems, one of which obtains to the occurrence of abnormality in different generations and the other to the duplication of the parental abnormality. Data pertinent to the first problem have been presented in this paper and show that the presence of abnormality without regard to its extent or location is dependent upon a simple recessive Mendelian factor. The second problem has been investigated and, while the details will be published elsewhere, the results may be briefly summarized for present purposes.

It was noted that an exact duplication of the location and extent of abnormality rarely occurred in the progeny of similarly affected animals and a series of interbreeding experiments was undertaken to determine whether or not these variables were under genetic control and to study the nature of the factors concerned. It was found that the extent and location of abnormality were determined by two groups of hereditary factors, which did not segregate in the manner of unit characters but behaved rather as quantitative modifying factors. The factors of the two groups are not entirely specific in influence and their action may be expressed under certain conditions on either the extent or location of abnormality. Thus, if the factors influencing the extent of abnormality are present in great concentration in parents a commensurate degree of abnormality is not necessarily expressed in progeny, but the factors may operate to produce abnormality in locations not affected in parents. The reverse is also true and location factors, in the event of a great concentration, may operate to increase the extent of abnormality. A further discussion of the genetic aspects of the abnormality will be deferred pending presentation of additional data.

The close association of functional deficiencies with the physical abnormality in the early history of the brachydactylous line suggested that they were parts of the same complex. Similar complexes involving the combination of a physical abnormality with functional disorders in apparently unrelated organ systems have been observed in the investigation of other hereditary variations and it was of interest to determine whether the functional alteration was an integral part of the condition or had been introduced into the line by a chance circumstance. The functional deficiencies disappeared after continued breeding of the line and it seems probable, at the present time, that the early combination was due entirely to a chance association.

The morphological changes observed in feti at various stages of gestation are similar in many respects to those described by Bagg in the descendants of x-rayed mice. The accumulation of lymph-like fluid that occurs in the early stages of the development of the abnormality in the mouse, and the thrombi described by Plagens (5), however, have not been found in the rabbit.

The first abnormal change noted in the rabbit is a dilatation of blood vessels without apparent alteration in the structure of their walls or of the nerves supplying the region. This is followed by extravasations of blood and necrosis of the tissue in the vicinity of the hemorrhage.

It is generally assumed in abnormalities of this type that the defect arises on the basis of inferior or faulty tissues. There is no evidence in the present instance that the involved tissues were morphologically different from adjacent tissues which remained unaffected. On the contrary, careful examination of many sections obtained at different periods prior to vascular dilatation failed to show any modification from normal.

Vascular dilatation has not been observed in other parts of affected feti, and the constancy of the finding in involved regions is highly suggestive of a direct causal relationship. The walls of blood vessels in affected regions are not morphologically altered, thrombi have not been observed, and no local cause has been found to account for the dilatation. In view of the absence of any demonstrable lesion, the possibility of an abnormal vasomotor condition is worthy of consideration. The absence of local constrictor fibers or the continued stimulation of existing fibers, giving rise to paralysis, would result in dilatation of the vascular segment, and subsequent stasis of the blood stream would be followed by hemorrhage and necrosis of the parts supplied by the segment.

A determination of the primary fault is not possible from the evidence at hand. It seems probable, however, that the somatic variation controlled by genetic factors was situated in vascular or nervous tissues. The subsequent course of events initiated by the primary variation proceeded in an orderly pathological sequence, giving rise to the expressed deformity in a manner entirely comparable to that which follows an abnormal environmental stimulus.

The preservation of blood cells and of epithelium in the necrotic areas is of further pathological interest. Hemolysis did not occur and the staining properties of extravasated red blood cells were unaltered, although the cells of surrounding tissues were completely disintegrated. The observations of Clark and Clark bear on this point (6). In a detailed study of the fate of extruded erythrocytes they found that the extruded cells were not caused to disintegrate by the tissue fluids and occasionally remained for a week or more before removal by lymphatic capillaries or by pigmented wandering cells.

Epithelium remained intact despite the absence of a blood supply, and in many instances active proliferation was observed in sloughed tissue fragments. Epithelial growth was apparently maintained by direct imbibition of nutrient from the amniotic fluid as in tissue culture. It seemed possible that proliferation in sloughed fragments might continue to the end of gestation and that the abnormal environmental relations might give rise to atypical changes, but gross particles have not been found at term, indicating that if the epithelium continued to live, proliferation was restricted and remained orderly.

SUMMARY

A series of deformities in the rabbit ranging from brachydactylia to acheiropodia have been described. Experimental breeding demonstrated that the occurrence of the deformities was determined by simple recessive hereditary factors and that the various types were genetically related and were not distinct hereditary entities.

Embryological investigation showed the first abnormal change was a dilatation of blood vessels in affected buds. This was followed by hemorrhage and necrosis of the parts involved. Sloughing subsequently occurred, and the deformity was completely expressed by the 25th day of fetal life.

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EXPLANATION OF PLATES

PLATE 15

FIG. 1. Young rabbit showing an almost complete absence of digits on all feet· The right ear contains a typical defect in its superior lateral margin.

FIG. 2. Adult rabbit showing a complete absence of the right hind foot, with varying degrees of brachydactylia in other feet.

FIG. 3. X-ray photograph of left front foot showing an absence of phalanges in the 2nd, 3rd, 4th and 5th digits. The remaining phalanx in the 2nd digit is the proximal phalanx. Frequently when a proximal phalanx acquires a terminal position in a digit due to the loss of other elements its distal extremity appears compressed and pointed, so that the form of the bone resembles that of an ungual phalanx.

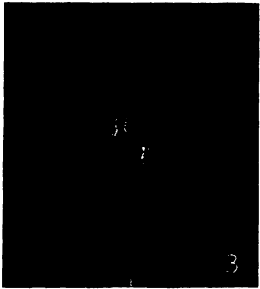
FIG. 4. X-ray photograph of right front foot showing an absence of phalanges in the 2nd, 3rd, 4th and 5th digits. Alterations near the articular surface are usually associated with dislocation of the affected or an adjacent phalanx.

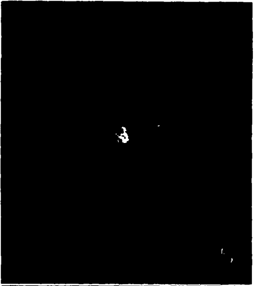
FIG. 5. X-ray photograph of left front foot showing an absence of all phalanges in the 2nd, 3rd, 4th and 5th digits, together with shortening and decrease in the diameter of the metatarsals.

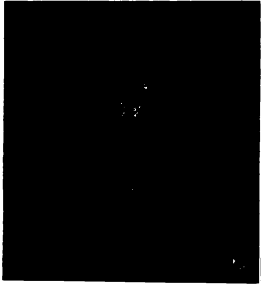
FIG. 6. X-ray photograph of left front foot showing an absence of all phalanges in the 2nd, 3rd and 5th digits, together with marked distortion of the metatarsals.

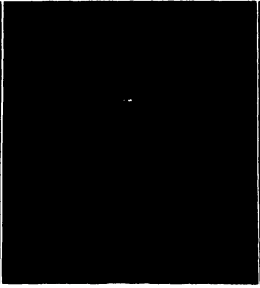
FIG. 7. X-ray photograph of right front foot showing an absence of all phalanges in the 2nd, 3rd, 4th and 5th digits, together with a reduction of the metatarsals to short pointed spicules.

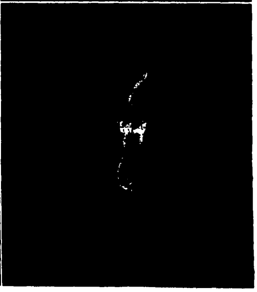
FIG. 8. X-ray photograph of the left hind foot showing an absence of all elements with the exception of the astragalus.











Photographed by J. A. Carlile

(Greene and Saxton: Hereditary brachydactylia)

PLATE 16

FIG. 9. Young rabbit showing the typical deformity involving the tips of both ears.

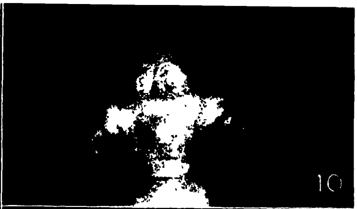
FIG. 10. 18 day old fetus showing red areas of discoloration on the tips of the ears. × 1.9.

FIG. 11. 19 day old fetus showing a swollen, sharply outlined area of deep red discoloration. × 1.9.

FIG. 12. Ears of 21 day old feti with control ears from normal animals of the same age. A line of demarcation has formed and the affected region is swollen and purple in color. × 2.7.

FIG. 13. 23 day old fetus. A line of demarcation has formed on the foot and the affected region is dry and black in color. × 2.7.

FIG. 14. 24 day old feti. The gangrenous parts of the feet have sloughed off and the stubs have healed. × 1.35.



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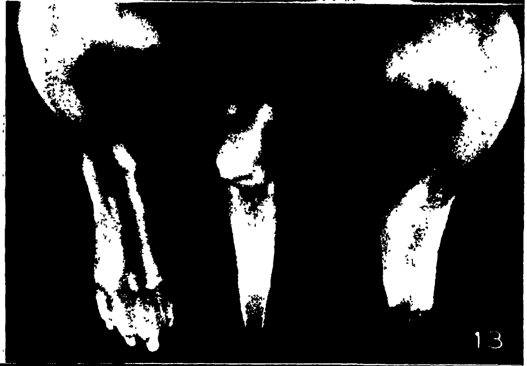

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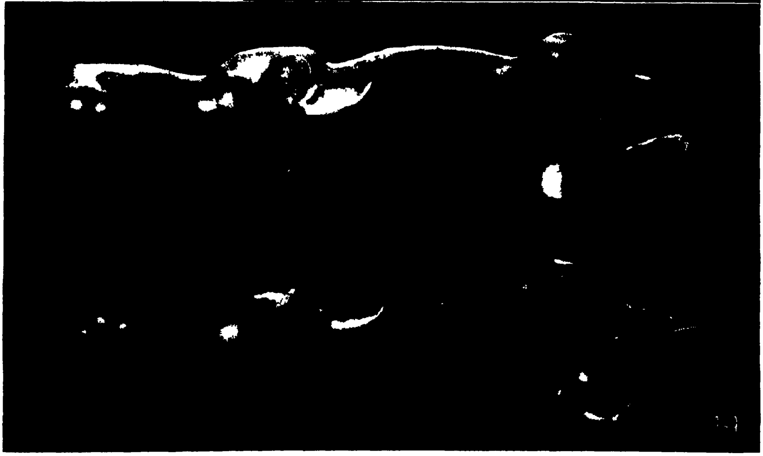

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Photographed by J. A. Carlile

(Greene and Saxton: Hereditary brachydactylia)

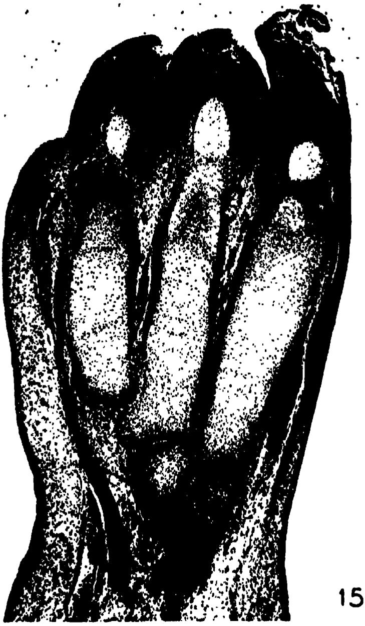
PLATE 17

FIG. 15. Section through the hind foot of a 21 day old fetus. Parts of the 2nd, 3rd, 4th and 5th digits are shown. The distal parts of the digits and the anlage of all except the proximal phalanges have been destroyed. Blood vessels are widely dilated. Hematoxylin and eosin. × 25.8.

FIG. 16. Section through the front foot of a 21 day old fetus. With the exception of the 1st digit and the metatarsal of the 5th, all the phalanges, metatarsals and soft parts are necrotic. Hematoxylin and eosin. × 25.8.

FIG. 17. Section through the front foot of a 20 day old fetus showing hematoma formation with necrosis in the distal portion of the 2nd digit. Hematoxylin and eosin. × 25.8.

FIG. 18. Section through the front foot of a 23 day old fetus showing hematoma formation with necrosis. The downgrowth of epithelium from the edges of the defect has separated the necrotic region from living tissue. Hematoxylin and eosin. × 25.8.



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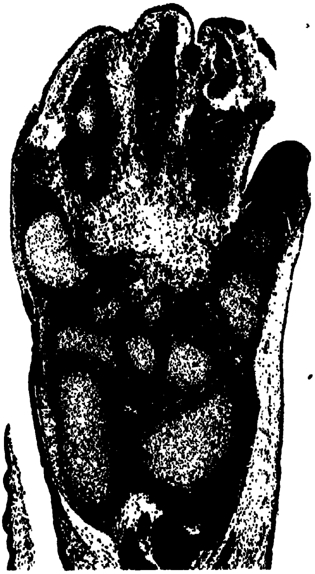

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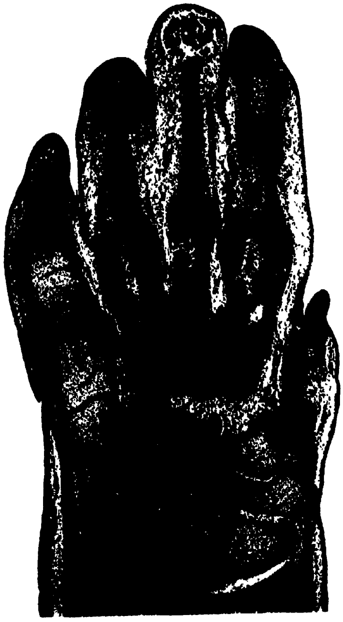

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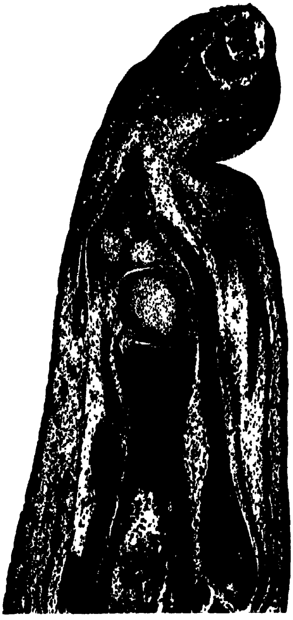

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Photographed by J. A. Carlile

(Greene and Saxton: Hereditary brachydactylia)

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AGE AND SEX DIFFERENCES IN HORMONE CONTENT OF THE RABBIT HYPOPHYSIS

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Constitutional factors of age and sex are known to influence the expression of many spontaneous and experimental diseases. In a study of these factors, attention has been directed to the endocrine system. The following experiments were performed to obtain an index of the normal age and sex differences in endocrine function in one of the commonly used laboratory animals, the rabbit. Since the anterior hypophysis occupies a dominant position in the endocrine system, studies were confined to this gland. Estimates were made of the relative content, according to age and sex, of certain anterior lobe hormones of the rabbit hypophysis, based upon changes found in the thyroids, ovaries and adrenals of immature guinea pigs following pituitary implantations.

Materials and Methods

The procedure was essentially that employed by Loeb in an analysis of anterior pituitaries of different species (1), and in a determination of differences in hormone content of the human anterior hypophysis dependent upon age and pregnancy (2). This method makes possible the analysis of individual hypophyses from mature rabbits, and small groups of glands from young animals. The results of preliminary experiments, using various amounts of tissue from mature male rabbits, were in agreement with the findings of Loeb for this species. Changes in the immature guinea pig ovary produced by gonadotropic hormones have been described in detail by Loeb (3).

All of the rabbits used were bred in a large animal colony, and the exact age and breed were known. Most of the animals used were hybrids, chiefly Belgian-English crosses, and a few Dutch and Himalayan hybrids. Others were of pure English, Belgian, and Dutch breeds. Including several supplementary experiments which were not tabulated, the pituitaries of 96 rabbits aged 10 days to 45 months were assayed, and 52 test animals were used, 46 of which received implants.

Pituitaries were obtained from freshly killed rabbits, immediately weighed, and kept on ice until used. Immature female guinea pigs, weighing from 170 to 210 gm. received subcutaneous implants of hypophyseal tissue daily for 4 days, and were killed for examination on the 5th day. Pituitaries from adult rabbits were analyzed by implantation of one quarter gland daily, and in the case of young rabbits, from one-half to two glands were used daily, depending upon their size. In this way the amounts of tissue implanted in each comparable series were kept fairly constant. The posterior lobe was not removed before implantation, and division of the whole gland always included a portion of it in each fraction. A preliminary experiment showed that the presence of the posterior lobe would not appreciably affect the responses of the test animals to anterior lobe hormones.

At autopsy both ovaries and the left adrenal of each guinea pig were weighed and the gross appearance of the thyroid, uterus and vagina was noted. Following fixation in Petrunkevitch's solution, sections 4.5 μ in thickness were cut through representative areas of both ovaries, the left adrenal, and one thyroid lobe, and stained with hematoxylin and eosin. Conclusions were largely based upon examination of these sections.

The presence of large and mature follicles, usually associated with increased weight of the ovaries, was taken as evidence of the follicle stimulating hormone. Enlargement of the uterus and proliferation of the vaginal mucosa offered further evidence of the presence of active mature follicles. Luteinization of the theca interna around growing or atretic follicles, formation of interstitial gland, and the development of pseudolutein bodies gave an index of the amount of luteinizing hormone in the implanted tissue. Since there is no standard measure for these effects, arbitrary values based upon microscopic comparisons are given in the results (tables 1 to 5).

Changes in the thyroid are similarly designated. The gross appearance of the gland was seldom altered and microscopic changes were usually slight. A single plus indicated a trace of hypertrophy, consisting in enlargement of some of the cells lining peripheral acini, together with the appearance of small vacuoles in the adjacent colloid. Higher values indicated greater activity.

Stimulation of the adrenal cortex was indicated partly by weight increase, but chiefly by increased numbers of mitoses. The mitotic figures in three representative sections in each case were counted.

TABLE 1

Controls: Gross and Microscopic Findings in 6 Untreated Guinea Pigs

Guinea pig No.	Weight	Uterus and vagina	Ovaries			Thyroid stimulation	Left adrenal	
			Average wt.	Maturation of follicles	Luteinization		Weight	Mitoses per section
	gm.		*mg.*				*mg.*	
6	180	Thin, vagina closed	7.5	0	0	0	35	2
9	204	Medium, vagina closed	21	0	0	0	50	—
72	185	Thin, vagina closed	19	0	0	0	47	19
73	190	Medium, vagina closed	17	0	0	0	34	12
122	175	Medium, vagina closed	11	0	0	0	36	6
138	200	Medium, vagina closed	22.5	0	0	0	42	12
Average		Medium to thin, vagina closed	16.3	0	0	0	40.7	10.2

In glands showing stimulation, mitoses were most frequent in the outer portion of the zona fascicularis.

RESULTS

a), *Controls.* Six normal female guinea pigs of the same weight and stock used in the experiments were examined at different times, and the findings are recorded in table 1. The uterus and vagina were not enlarged in any of the cases, and the vagina was invariably closed. There was no gross or microscopic evidence of sexual maturity. The ovaries weighed from 7 to 27 mg. Microscopic examination showed the presence of growing follicles and a few large follicles which did not reach maturity in any case, but appeared to undergo atresia after reaching large size. The thyroid was never enlarged, and there was no microscopic evidence of stimulation. Rather flat epithelium

TABLE 2

Implantation of Hypophyses from Young Adult Male Rabbits

Exp. No.	Rabbits used (all ♂)		Effects on the Guinea Pigs							
	Breed and age	Wt. of pituitary	Initial and final wt. of test animals	Condition of uterus	Ovaries			Thyroid stimulation	Left adrenal	
					Average wt.	Follicle maturation	Luteinization		Wt.	Mitoses per section
		mg.	*gm.*		*mg.*				*mg.*	
131	Belg.-Eng. 4 mo.	44	190–170	Enlarged vag. open	57	+++	++++	+	47	50
132	Hyb.-Eng. 4 mo.	26	185–175	Enlarged vag. open	116	++++	++++	+	73	68
133	Eng.-Hyb. 4 mo.	29	175–170	Enlarged vag. open	78	++++	++++	0	54	69
136	Belg.-Eng. 4 mo.	30	190–205	Medium vag. closed	33	±	+++	++	72	56
137	Belg.-Eng. 4 mo.	29	180–180	Enlarged vag. closed	51	+++	++++	+	73	39
143	Dutch 4½ mo.	27	185–210	Enlarged vag. open	34	++	++++	+	74	85
189	Belg.-Eng. 5½ mo.	23	170–185	Enlarged vag. open	77	++++	++++	+	54	23
190	Belg.-Eng. 5½ mo.	25	165–180	Enlarged vag. open	37	++±	+++	++	51	29
161	English 5½ mo.	21	185–180	Enlarged vag. open	78	++++	+++±	+	60	86
159	English 6 mo.	21	180–185	Enlarged vag. open	66	++++	+++	+	52	41
Average		27.5			62.7	+++	+++±	+	61	55.6

TABLE 3

Implantation of Hypophyses from Young Adult Female Rabbits

Exp. No.	Rabbits used (all ♀)		Effects on the Guinea Pigs							
	Breed and age	Wt. of pituitary	Initial and final wt. of test animals	Condition of uterus and vagina	Ovaries			Thyroid stimulation	Left adrenal	
					Average wt.	Follicle maturation	Luteinization		Wt.	Mitoses per section
		mg.	*gm.*		*mg.*				*mg.*	
105	Belg.-Eng. 5 mo.	42	175–165	Enlarged vag. open	22	+±	++±	++	53	34
106	Belg.-Eng. 5 mo.	32	175–155	Enlarged vag. open	37	++±	++	+	60	20
110	Belg.-Eng. 5 mo.	38	180–165	Mod. enlarged vag. open	33	+++	++++	0	45	17
111	Belg.-Eng. 5 mo.	40	185–190	Enlarged vag. open	25	++	++	++	50	16
114	Belg.-Eng. 5 mo.	—	185–185	Enlarged vag. open	19	++	+++	+	53	6
194	Belg.-Eng. 5 mo.	54	190–195	Mod. enlarged vag. closed	27	+±	++±	0	50	29
192	Belg.-Eng. 5 mo.	40	170–195	Enlarged vag. closed	37	++	++++	++	66	13
119	Belg.-Eng. 5½ mo.	30	190–200	Enlarged vag. open	32	++	+++	+	59	20
162	English 8 mo.	33	185–205	Enlarged vag. open	18	+±	+++±	+	50	134
158	English 8 mo.	35	180–190	Enlarged vag. open	43	++±	++±	0	59	37
Average		38			29.3	++	+++	+	54.5	31.6

surrounded solid colloid in peripheral acini. The left adrenals weighed from 34 to 50 mg., and mitoses per section varied from 2 to 20. In view of the uniform condition of these animals, the average values for this series were considered as normal standards of comparison for the effects produced by the implantations.

b), *Effects of hypophyses from young mature rabbits of both sexes.* Hypophyses from 10 male and 10 female rabbits were analyzed separately and the values obtained in each experiment are shown in tables 2 and 3. The rabbits for the most part were English and Belgian-English hybrids aged from 4 to 8 months. On the average the males were slightly younger than the females, but in each instance sexual maturity was confirmed at autopsy. Virgin females were used, and none of the series showed evidence of pseudopregnancy when killed. The ovaries of these rabbits contained large clear follicles but no corpora lutea, and the uteri were usually in estrus condition. The males had not been used for breeding.

On the average, the pituitaries from female rabbits produced moderate gonadotropic effects. The ovaries of the test animals were enlarged, and usually contained two or three large follicles, together with a few smaller growing follicles. The interstitial gland was moderately well developed and a corresponding degree of luteinization of the theca interna about growing and atretic follicles was observed. One or two pseudolutein bodies were found in 5 of the 10 cases. Follicular atresia was not more prominent than in the normal immature guinea pig ovary.

Implants of male pituitaries, despite smaller size, produced more intense gonadotropic effects. The ovaries of the test animals were greatly enlarged, and usually contained several mature follicles as well as growing follicles of all sizes. Luteinization of the theca interna of growing and atretic follicles was quite advanced, and the interstitial gland was well developed. Several pseudolutein bodies were present in 7 out of 10 cases.

Slight thyrotropic effects were noted in the majority of each group, and on the average there appeared to be no differences according to sex. From the tables it is apparent that a wide variation was obtained in the values for adrenal cortex stimulation. Male pituitaries, however, tended to produce a greater increase in adrenal weight and number of mitoses per section.

c), *Effects of hypophyses from immature rabbits of both sexes.* In this series 12 experiments were carried out. The hypophyses from small groups of rabbits of the same age, usually of the same sex, were analyzed together. Ages of the groups ranged from 10 to 73 days, and daily implants varied from 2 to ½ glands, depending upon size. The majority of the rabbits were Belgian-English hybrids, and litter mates were used whenever possible. A few were Dutch and Himalayan hybrids. Table 4 gives the values obtained in each experiment, and the sex of the rabbits used.

It will be noted that at 10 days of age the rabbit pituitaries exerted no gonadotropic effects, although some thyroid and adrenal cortex stimulation were produced. In addition to thyroid and adrenal stimulation, glands from 15- and 21-day-old rabbits produced some growth and maturation of follicles, although not in so great a degree as when glands from older immature rabbits were implanted. Pituitaries from 28-day-old rabbits exerted in addition a weak luteinizing effect, and glands from older animals up to 10 weeks of age produced both follicle stimulation and luteinization, more or less in proportion to increasing age. Luteinization processes, even following implants of 10-week-old glands, were not so intense as when glands from mature animals of either sex were implanted, and pseudolutein bodies were not found in any of the test animals of this group. When pituitaries from 4- to 10-week-old animals were implanted, growth and maturation of follicles were more marked than following glands from mature females, although in the absence of a comparable degree of luteinization, this apparent difference may not be significant. Where sexes can be compared, the male pituitaries were slightly smaller, and tended to produce somewhat greater gonadotropic effects. In all but two experiments the thyroid was stimulated, and on the average a greater thyrotropic effect was found than following implants of adult glands. Some adrenal stimulation was produced in each case, but was not correlated with age or sex.

d), *Effects of hypophyses from old female rabbits.* The hypophyses from 10 old female rabbits were analyzed in this group. The rabbits

TABLE 4

Implantation of Hypophyses from Immature Rabbits

Exp. No.	Rabbits used			No. of pituitaries implanted daily	Effects on the Guinea Pigs							
							Ovaries				Left adrenal	
	Age	Sex	Average wt. of pituitary		Initial and final wt. of test animals	Condition of uterus and vagina	Average wt.	Follicle maturation	Luteinization	Thyroid stimulation	Wt.	Mitoses per section
	days		*mg.*		*gm.*		*mg.*				*mg.*	
175	10	♂ & ♀	4	2	170–190	Medium vag. closed	16	0	0	+	60	27
129	15	♂ & ♀	6	1	190–195	Enlarged vag. closed	23	+±	0	+	48	12
128	15	♂ & ♀	6	1	195–200	Enlarged vag. open	30	++±	0	++	65	20
178	21	♂	7	1½	180–195	Enlarged vag. open	22	+	0	+	78	52
179	21	♀	7	1½	185–205	Moderately enlarged vag. closed	14	±	0	++	62	32
78	28	♂	10	½–1	185–165	Enlarged vag. open	37	++±	+	+	70	31
23	35	♂ & ♀	12	1	190–190	Enlarged vag. open	75	++++	+±	0	58	27
99	41	♂ & ♀	14	½–1	188–170	Enlarged vag. open	53	+++	++	0	42	21
169	52	♂	15	½	175–175	Enlarged vag. open	56	+++±	++±	++	60	67
168	52	♀	18	½	170–160	Enlarged vag. open	52	+++	++	++	51	16
167	73	♂	19	½	175–160	Enlarged vag. open	62	++++	++	++	80	16
166	73	♀	23	½	182–170	Enlarged vag. open	44	+++	++±	++	45	18

TABLE 5

Implantation of Hypophyses from Old Female Rabbits

Exp. No.	Rabbits used (all ♀)		Effects on the Guinea Pigs							
	Breed and age	Wt. of pituitary	Initial and final wt. of test animals	Condition of uterus and vagina	Ovaries			Thyroid stimulation	Left adrenal	
					Average wt.	Follicle maturation	Luteinization		Wt.	Mitoses per section
		mg.	gm.		mg.				mg.	
97	Belg.-Eng. 23 mo.	—	160–180	Enlarged vag. open	31	++	++	+	44	23
82	Belg.-Eng. 23 mo.	—	180–175	Enlarged vag. open	21	++	++±	+	58	29
123	Belg.-Eng. 23 mo.	—	200–200	Enlarged vag. open	26	+±	++	0	65	157
124	Belg.-Eng. 25 mo.	—	185–195	Enlarged vag. open	37	+±	++±	0	55	58
121	Belg.-Eng. 25 mo.	—	175–180	Enlarged vag. open	21	++	+++	0	49	16
60	Belg.-Eng. 26 mo.	—	175–165	Enlarged vag. closed	50	+++	++++	+	57	131
181	English 35 mo.	48	190–205	Enlarged vag. open	54	+++	+++	±	78	24
44	English 35 mo.	—	175–185	Enlarged vag. closed	26	+	++±	0	54	9
41	English 36 mo.	—	180–190	Enlarged vag. open	23	++	+±	0	60	108
35	Belg.-Rex. 45 mo.	45±	185–188	Enlarged vag. open	28	++±	++++	0	65	59
Average					31.7	++	+++	±	58.5	61.4

belonged to the same strains as the younger mature females and were aged from 23 to 45 months. In all instances the animals had been intensively bred and had subsequently been infertile for periods of about 6 months prior to study. They were neither pregnant nor pseudopregnant when killed. The ovaries usually contained a few large follicles and the uteri in most cases were grossly similar to those of the younger females. On the basis of breeding history, the animals were considered to be reaching the end of the period of reproductive activity. In 2 cases where the hypophyses were weighed, the glands were heavier than those of younger mature females. The others were similar in size or occasionally somewhat larger.

The results of implantations are shown in table 5. It will be seen that the gonadotropic potency was approximately equal to that of the younger mature females. Growth and maturation of follicles, changes in the theca interna of growing and atretic follicles, and the development of interstitial gland were quite comparable, but in only 3 cases were any pseudolutein bodies produced.

The thyroid was affected in only 4 of the test animals, and in these instances stimulation was relatively slight. Adrenal stimulation occurred in all but one case, and was usually of a higher order than found after implanting glands from younger mature rabbits, although individual variation was greater. A comparison with the effects of pituitaries from old male rabbits was not possible.

DISCUSSION

The rabbit hypophysis is well suited to studies of gonadotropic hormone content by this method. The proportions of gonadotropic hormones in glands of this species are such that implantation of small amounts of tissue (one-quarter gland daily from mature animals) permits the expression of both follicle stimulation and luteinization. Larger implants produce a predominantly luteinizing effect, masking to some extent the effects of follicle stimulating hormones. By way of contrast, the proportions of gonadotropic hormones in the adult human and bovine hypophysis are such that pure follicle maturation is seldom produced by implants of fresh tissue, regardless of size. Additional procedures, such as treatment with formalin or urea (4), or serial implantations (5), are necessary to bring out the complete follicle stimulating effects.

However, implants of the optimum size for gonadotropic effects produced a minimum of thyroid stimulation. This necessitated microscopical comparisons in all cases, and occasional supplementary experiments using larger implants were necessary to confirm questionable differences. In view of the sensitivity of the guinea pig adrenal to extraneous factors, all of the changes found in this gland can not with certainty be attributed to the action of a specific hormone. There is much evidence that the anterior pituitary has a stimulating effect on the adrenal cortex, independent of gonadotropic and thyrotropic effects (6), but the relationships are not at present sufficiently clear to warrant discussion.

These experiments present in a general way a composite picture of the content of certain hormones of the rabbit hypophysis, which differs both with respect to sex and age. Minor variations within the groups may in part be attributed to breed differences in the rabbits. While it was not possible to keep this factor constant in all cases, related breeds showing similar body characters were largely used. Preliminary studies have indicated that breed differences in pituitary hormone content may exist, but the age and sex differences described in this paper have been consistent in the breeds thus far studied.

The significance of the observed sex differences is obscure. In adults the proportion of follicle stimulating to luteinizing hormone seems to be approximately the same in the two sexes, the male pituitary having greater absolute quantities of each factor. It seems probable that we are dealing with differences in reproductive physiology, the nature of which is not suggested by this approach. These differences are manifest to a slight degree even in the immature rabbit, and it is of possible importance in this connection that the male rabbit has been found to reach maturity at a slightly earlier age than the female. Similar sex differences in gonadotropic hormone content have been noted in the rat by Evans and Simpson (7).

Differences between the mature and immature rabbit hypophysis are comparable to those observed in the case of the human hypophysis

(2). In both species gonadotropic hormones are present in relatively small amounts before sexual maturity, whereas the thyrotropic hormone is present in equal or perhaps greater concentration at this time. This age difference in the rabbit is further accentuated by the use of larger implants. In 4 supplementary experiments one gland daily from 4- and 7-week-old rabbits was implanted. A very marked thyroid hypertrophy resulted in each instance, but the ovaries of the test animals weighed only from 7 to 27 mg., compared with 37 to 75 mg. when half this amount of tissue was used. The degree of thyroid hypertrophy was even greater than found after implanting comparable amounts of adult tissue, which invariably produced greater follicle stimulation and luteinization. Large implants of young glands therefore seemed to inhibit growth and maturation of follicles.

To explain this inhibition, two possibilities are suggested. In the first place, an antagonism may exist between the thyroid and follicle stimulating hormones. However, it has been shown that implantation of pituitaries from children may bring about growth and maturation of follicles together with a marked thyroid hypertrophy, so that an antagonistic relation between these hormones does not necessarily exist. It is more probable, therefore, that another factor, such as "atresin," described by Loeb in the bovine hypophysis (8), is present in the rabbit hypophysis and that its action is manifest under these conditions. Atresia of follicles was not observed, but this factor may merely inhibit growth and development of follicles.

The results of implanting glands from older mature rabbits indicate that there is no appreciable change in the amounts of gonadotropic hormones with increasing age past sexual maturity. Similarly, human hypophyses showed no changes in this respect. There was, however, in the case of rabbits a tendency for the thyrotropic hormone content to diminish with age, and for the adrenal stimulating factor to increase.

SUMMARY

Age and sex differences in hormone content of the rabbit hypophysis have been studied by means of implantation into immature female guinea pigs.

The male pituitary produced greater follicle stimulation and luteini-

zation than did the female, and also a greater stimulation of the adrenal cortex. Thyrotropic effects were approximately equal. Pituitaries from 10-day-old rabbits produced thyroid and adrenal stimulation but no gonadotropic effects, whereas at 15 and 21 days follicle stimulation was also produced. Glands from 28-day and older rabbits produced both follicle stimulation and luteinization, although of a moderate degree. The thyrotropic effects of comparable amounts of pituitary tissue from immature rabbits were greater than from mature rabbits. Glands from old female rabbits produced about the same degree of follicle stimulation and luteinization as young mature females. However, there was less thyroid stimulation and generally greater stimulation of the adrenal cortex.

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UTERINE ADENOMATA IN THE RABBIT

II. HOMOLOGOUS TRANSPLANTATION EXPERIMENTS

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PLATES 28 AND 29

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The occurrence and course of spontaneous adenomata and adenocarcinomata of the uterus in the rabbit and the successful transplantation of one of the tumors to animals of the same species were reported in a previous paper (1). Investigation of the transplanted tumor has been continued and extended in various directions with a view to determining the essential biological and pathological characteristics of the growth as compared with other transplantable tumors now used for experimental purposes.

Many spontaneous tumors have been reported in the rabbit but very few have been successfully transplanted to normal animals. With the exception of the Brown-Pearce epithelioma (2) all of the transplantable tumors have been sarcomata and with the further exception of Kato's sarcoma (3) all were lost before any extensive experimentation could be carried out. During the past year, three different types of spontaneous rabbit carcinomata, in addition to the uterine tumor, have been successfully transplanted and are being studied in a similar manner to supply a broader basis for comparison and as additional agents for the investigation of cancer problems. It has been found that the uterine tumor, designated as H31, is manifestly different from other transplanted rabbit tumors and possesses certain potentialities which render the growth a more advantageous medium for the study of certain phases of the tumor problem.

The object of the present paper is to report the results of several series of interrelated experiments including: first, serial transplantation in normal rabbits; second, transplantation in rabbits with spontaneous tumors of the same nature; third, reinoculation of animals with a growing transplanted tumor; fourth, reinoculation of animals which proved refractory to an earlier attempt at transplantation and of animals in which transplanted tumors had completely retrogressed and finally, a series of experiments in which frozen and dried tumor material was introduced into normal uteri.

While this report must be limited to results obtained with the uterine tumor referred to above, comparative statements of results are based in part upon experience with other tumors. Details of these experiments will be reported later.

Materials and Methods

The technique of transplantation employed in the present experiments has been fully described elsewhere (1). Whole fragments of tumor tissue measuring approximately 1 mm. in diameter were used in transfers to the anterior chamber of the eye, while testicular inoculations were made with 0.3 cc. of a thick cellular emulsion.

The present report is based on a clinical and pathological investigation of the tumor through 12 serial generations in the anterior chamber of the eye and 6 serial generations in the testicle. Tumor tissue was transferred to the eyes of 23 normal males and 62 normal females while 95 males were used for testicular inoculation. The results of simultaneous inoculation of both testicles or of one testicle and an eye were studied in 5 animals. Transplants were also made in 8 females with spontaneous uterine tumors and 13 animals in which the tumor had failed to grow were reinoculated after periods of 35 and 69 days following the first inoculation. In addition, transplants were made in 3 animals in which a previously transplanted tumor was actively growing and in 13 animals in which the previously transplanted tumor had completely retrogressed.

Six animals were used in an attempt to induce neoplasia with dried frozen tumor material. Fresh tumor tissue was obtained from the eye of an animal of the 6th serial generation. The tissue was immediately frozen in dry ice and dried in a vacuum machine. The resulting powder was emulsified in water and injected directly into the uterine mucosa. The animals were killed 42 days after injection and sections of the mucosa were examined histologically.

Throughout the experiments particular attention was directed toward a thorough gross and histological postmortem examination, not only to follow the morphological characteristics of the tumor and to discover the presence or absence of metastases but, also, to determine the condition of those organs altered in spontaneous cases of the tumor. Pituitary glands were fixed in Susa's solution and stained by a modification of Mallory's aniline blue method, while routine tissues were fixed in Petrunkevitch's solution and stained with hematoxylin and eosin.

Serial Transplantation into the Anterior Chamber

The results of serial transfer of tumor fragments into the anterior chamber of the eye are presented in Table I. Successful transplantaion was effected in 82.8 per cent of all the animals used but varied from 33.3 per cent to 100 per cent in different generations. In early transfers the tissue was obtained from transplants varying from 2 to 3 months in age while in later transfers the interval between transplantations rarely exceeded 1 month.

TABLE I

Anterior Chamber Series

Generation	Date of transfer	Number of animals used	Takes
	1937		*per cent*
1	Mar. 10	3	66.6
2*a*	May 18	3	33.3
2*b*	" 28	3	66.6
3	Aug. 10	6	33.3
4	Oct. 21	8	87.5
5	Nov. 29	8	87.5
	1938		
6	Jan. 13	7	100.0
7*a*	Feb. 24	6	83.3
7*b*	Mar. 3	6	50.0
8*a*	Apr. 5	3	100.0
8*b*	" 12	14	100.0
9	May 18	7	100.0
10	June 20	5	100.0
11	July 20	8	75.0
12	Sept. 21	6	100.0
Total........		93	82.8

The age of the transplant undoubtedly played a part in the greater incidence of takes but there is also evidence that adaptation to the environment of the anterior chamber was of considerable importance in this respect. Two experiments bear on this point. In one experiment tissue fragments from a growth in the testicle representative of the 7th generation of the tumor were transferred to the anterior chambers of 14 animals and resulted in 38.5 per cent of takes. In a second experiment the transfer of fragments of another testicular growth of the same generation into the eyes of 13 animals resulted in 41.6 per cent of takes. These results are similar to those observed in the first part of the series under discussion, despite the fact that the tumor had been successfully transplanted for 7 generations, and indicate that the high percentage of takes in the latter part of the series was influenced by adaptation to the environment of the anterior chamber, rather than by adaptation to transplantation in general.

The regular increase in the incidence of takes was interrupted in August, 1937, and in July, 1938. A similar reduction in the number of takes during these months has been noted in the study of other tumors propagated by different routes of inoculation and has occurred to a much greater degree in the testicular series of the present tumor.

Latent Period.—A gradual decline in the period required before growth of the transplanted fragments became apparent was observed throughout the first 5 generations but, even after the 5th transfer, definite evidence of growth could only rarely be detected in less than 1 month. Abruptly after the 6th transfer this period was cut to 14 days and while, at the present time, signs strongly indicative of growth may be found in a shorter period, an earlier positive diagnosis of growth cannot be made.

The first indication of survival is a pinkish color change in the transplanted fragment without any appreciable increase in its size. At the present time, this can often be detected by the 6th day. The color change appears simultaneously in all parts of the fragment and increases slightly in intensity throughout the following week. In contrast, fragments that fail to grow become dull white in color and opaque. In recent transfers, minute pin point pinkish-white areas are frequently observed in regions where formerly no trace of the transplant could be seen. Such areas have been interpreted as resulting from the growth of cells dislocated from the graft during its passage through the chamber.

An increase in the size of the transplant can usually be detected by continuous observation during the 2nd week. This is facilitated by the transplantation of fragments with irregular outlines so that small changes in a given locus can be readily perceived. Comparative drawings taken at daily intervals, in such cases, show a slight but definitely detectable tendency toward the rounding out of sharp, angular irregularities which is usually apparent by the 8th day. This process continues and at the time of vascularization, the irregular outlines have disappeared and the transplant is round or oval in shape.

Vascularization and Subsequent Growth.—The transferred tumor fragments always become attached to a fixed part of the anterior chamber, usually the iris, within 12 hours of transplantation. Attachment occurs whether or not growth subsequently takes place and is evidently brought about by an exudative reaction on the part of the host. It is not associated with a connective tissue or vascular proliferation but is loose and may be disrupted by applying pressure along the corneal surface. Evidence of vascularization has not been detected before the 14th day and is frequently not observed before the 21st day. It should be noted, however, that stroma replacement and vascularization began along the area of attachment and the process may be of some duration before blood vessels can be seen in the gross on the exterior of the transplant.

In all but a single instance, fragments that have undergone the color change and increase in size previously described have eventually become vascularized. Vascularization is often delayed for as long as 3 weeks and in the interim the fragment may double its mass or remain without appreciable change. In exceptional cases, fragments have persisted without a detectable blood supply or increase in size for 2 months and then have become vascularized with subsequent rapid development into large tumors.

Vascularization is effected by a growth of vessels from the iris which permeate the graft in all directions and form a fine, complicated network about the growing edge. The appearance of the vascularized transplant is sufficiently characteristic to differentiate it from grafts of other tumors of different origin growing in the same environment. The tissue appears homogeneous and pale pinkish-white in color, except for the peripheral region which is of a deeper hue. Occasional minute greyish flecks are seen but the patterned arrangement of dark and lighter areas found in other growths is entirely absent. The tendency to form rounded masses with smooth clear-cut edges persists until infringement on the boundaries of the anterior chamber forces a change of shape.

Eventual Fate.—A large proportion of the animals used in these experiments were killed as soon as successful transplantation into the next serial generation became apparent, but others were held to determine the eventual fate of the growth.

The growth rate was increased after vascularization in all instances but varied in different generations and in individual animals. The period required for the complete filling of the anterior chamber varied from 45 to 105 days in animals of the same generation and, while the chamber was rarely filled in less than 60 days in the earlier transfers, complete replacement has been observed by the 20th day in recent generations. It should be noted, however, that in recent generations, growth invariably occurred in multiple foci and the resulting nodules coalesced so that the increase in growth rate was not as great as is suggested by these time relations.

After filling the anterior chamber, the growth underwent regressive changes in approximately 70 per cent of cases. The tissue became brown, granular and opaque and, eventually was entirely resorbed leaving no permanent damage other than large corneal scars and occasional synechiae. On the other hand, in the remaining 30 per cent of cases, growth continued, the cornea was invaded and the tumor protruded externally as a large fungating mass. Animals of this class have been killed for humane reasons, and hence there is no telling what their eventual fate would have been. Autopsy revealed the presence of metastases in two instances. One animal had been killed on the 184th day after transplantation and secondary growths were found in the regional lymph nodes, the lungs, the pancreas and the left ovary. In another animal killed on the 161st day, the regional nodes alone were involved.

As a rule, the more rapidly growing transplants undergo regression after filling the chamber, while those with a slower growth rate tend to invade and to extend to the outside. It seems probable that the regressive changes may be a direct result of an increased intraocular pressure incident to rapidly expanding growth in a confined space and leading to a progressive diminution of blood supply. The fact that, in the normal course of events, regression has never been observed before the entire chamber is filled is also suggestive in this respect. It is of interest in this connection that surgical interference with removal of a small part of the graft is almost invariably followed by regression.

No attempt was made in the present series of experiments to select animals to test the effect of different constitutional factors on the susceptibility to transplantation or on the eventual fate of the growth. Hybrids were used, for the most part, and no indication of breed differences can be obtained from the data. An analysis of the results on an age basis shows that while no significant variation in the susceptibility to transplantation occurred, the transplants grew more rapidly in young animals and after replacing the anterior chamber, regression rather than continued extensive growth was the rule. The percentage of takes was the same in males and in females and the subsequent fate of the tumor was not altered by the sex of the animal.

Histological Examination.—Histologically, the transplants obtained from animals of early serial generations showed an approximate duplication of the characteristics of the parental tumor (Fig. 1). There was an abortive attempt at the formation of acini which for the most part were composed of solid cellular masses and were without a lumen. Mitotic figures were not numerous and degenerative changes were rarely found. The stroma was abundant and myxoid in character.

In later generations, growth was more atypical and anaplastic (Fig. 2).

A tendency to form rounded masses was still apparent, particularly along the advancing edge of the tumor, but in other regions individual masses had coalesced to form solid sheets. In such regions the frequent occurrence of round areas of necrosis surrounded by concentrically arranged epithelial cells indicated the manner of growth. Stroma was sparse and poorly differentiated. Large cellular regions were separated by fibrous connective tissue extensively invaded by epithelial cells which formed abortive acini in some areas but grew, for the most part, in short strands and columns giving the tissue a marked resemblance to sections of scirrhous carcinoma. The number of mitotic figures and the amount of necrosis were directly proportional to the rate of growth observed clinically.

Necrosis dominated the picture in animals killed while the tumor was undergoing clinical regression. Large circumscribed necrotic areas were distributed focally throughout the cellular regions and in older growths these regions were completely necrotic except for a narrow rim of intact cells surrounding vascular channels. On the other hand, epithelial cells enmeshed in fibrous tissue remained intact for a longer period of time and the eventual disintegrative changes proceeded through karyorrhexis rather than karyolysis as in the more cellular areas.

All structures of the anterior chamber with the exception of the lens were invaded and destroyed in the large fungating types of growth. The posterior chamber was frequently involved and was occasionally found filled with tumor, but extension through the sclera to retro-orbital tissues has not been observed.

Metastases were less cellular in structure than the primary growth and the connective tissue reaction was more marked (Figs. 3 and 4). The proliferation of fibrous connective tissue was so intense in the lung, particularly near the pleural surface, that serial sections were frequently required before the essential epithelial elements could be found. The resemblance to scirrhous carcinoma was striking in these regions but in other organs parenchymal cells were grouped in atypical acinar arrangement and the stromal relations approximated those of the primary growth.

Serial Transplantation in the Testicle

Transplantation of the tumor into the testicle was successfully carried out from the 3rd, 4th and 5th serial eye generations and the growth obtained from the 4th eye generation has been propagated by continued serial transfer in two different lines of animals.

The results of serial transfer by this route of inoculation are presented in Table II. Growth occurred in only 41.05 per cent of the animals used and the success of inoculation was extremely irregular in the different serial generations. 100 per cent of takes was obtained at one transfer while other transfers resulted in complete failure. The more successful transfers were made during the fall and winter months and the largest per cent of takes resulted from inoculations made in February and early March. On the other hand, the failures occurred in the late spring and summer.

TABLE II

Testicular Series

	Testicular generation	Serial generation of tumor	Date of transfer	Number of animals used	Takes
			1937		*per cent*
Series A	1	4	Oct. 21	2	50.0
			1938		
	2	5	Mar. 3	4	75.0
			1937		
Series B	1	5	Nov. 29	3	66.6
			1938		
	2	6	Jan. 12	8	25.0
	3	7	Mar. 3	3	100.0
	4	8	Apr. 5	9	66.6
	5	9	June 13	4	75.0
	6	10	July 21	8	25.0
	6	10	Aug. 22	7	00.0
Series B_1	3	7	Feb. 16	11	54.5
	3	7	Mar. 3	10	50.0
	4	8	Apr. 4	12	25.0
	5	9	May 28	5	00.0
	5	9	July 13	4	00.0
Series C	1	6	Feb. 4	5	80.0
Total..............				95	41.05

A distinction between the effects of adaptation and of season on the behavior of the testicular transplants is extremely difficult in the present series. Extreme seasonal variations are known to occur and the present series of experiments were undertaken at different seasons. Thus, while the success of the first generation transfers appears to be directly related to the length of time that the tissue used had previously been serially transplanted in the eye, there is no indication in the data as to whether season or adaptation to transplantation was the determining influence. It is evident, however, that the percentage of takes in subsequent testicular generations was not increased by continued passage in that organ.

The percentage of takes was not consistently increased by shortening the interval between transfers but here again the influence of season may have been operative. On the other hand, the latent periods in the first generation transfers averaged 30 days irrespective of the season in which inoculations were made. Moreover, this period decreased to 14 days in subsequent generations and was not altered with changes of season.

The rate of growth varied within wide extremes but the variations were more marked between animals of different genetic constitution in the same generation than between similar animals in different generations. In some animals, the growing nodule never progressed beyond the size of a pea and was morphologically distinguished by an intense connective tissue proliferation and an acinar arrangement of epithelial cells (Fig. 5). On the other hand, the majority of animals developed multiple nodules which grew rapidly and completely replaced the testicle by the 40th day after inoculation. Histologically, such tumors resembled the rapidly growing transplants in the anterior chamber and were characterized by large confluent cellular masses with centrally placed areas of necrosis (Fig. 6). Growth was both expansive and extensive in character, destroying testicular parenchyma both by pressure and by active infiltration.

Infiltrative growth was limited to the testicular parenchyma for a long period of time and extension to the tunica vaginalis or to the spermatic cord was not observed before the 160th day. Expansive growth continued and eventually with encroachment on the blood supply, fluctuating necrotic areas appeared. Occasionally the entire testicle was converted into a sac distended with black fluid necrotic material, but even in such instances active nodules of growth were found on histological examination. In the majority of cases, however, such degenerative changes were limited to small areas and connective tissue replacement rather than necrosis characterized the older growths. The connective tissue was, in turn, invaded by tumor cells growing in strands and in isolated acinar groups. Growths of this type reached a large size and after the 160th day the testicle frequently measured 7 × 5 cm. and was characterized clinically by a firm nodularity. The eventual outcome of such cases is not known. One animal killed

on the 216th day showed fibrosis of the testicle with a complete destruction of all tumor cells, while the growth in another animal held for 240 days is still increasing in size.

Metastases have been found in three instances. The lymph nodes of the mesentery of the large intestine were involved in one animal killed on the 90th day and in a second animal killed on the 113th day which, in addition, showed a large metastatic nodule in the substance of the diaphragm. The third animal was killed on the 233rd day and secondary growths were found in nearly all organs of the body (Fig. 7). Microscopically the structure of the secondary growths was similar to that of metastases arising from transplants in the anterior chamber.

Simultaneous Transfers to Different Sites

Bilateral growths have been obtained from the simultaneous inoculation of both testicles in the same frequency with which inoculations into a single testicle have proved successful. Simultaneous transfers to the testicle and to the eye have resulted in growth in the eye alone in three instances and in growth in both locations in two instances. It is of interest that in one of the latter cases a metastatic growth was found at autopsy in a lymph node of the anterior triangle of the neck, while metastases were not found in the drainage area of the testicle.

Reinoculation of Refractory Animals

Transplantation of the tumor into the testicles following a primary unsuccessful inoculation has been attempted in 13 animals. The same testicle was used in the second attempt throughout the experiments. Control inoculations were made and gave a high percentage of takes.

Reinoculation of 12 of the animals was performed 35 days after the primary failure and was unsuccessful in all instances. On the other hand, reinoculation was delayed for 69 days in one animal and resulted in a take.

Transplantation into the Anterior Chamber of Animals with Spontaneous Uterine Tumors

The transfer of tumor fragments into the anterior chambers of 8 animals bearing spontaneous tumors of the same nature resulted in 5 takes, an incidence of about 60 per cent in contrast to an incidence of approximately 80 per cent in normal animals. The transfers were made coincidently with the 1st, 2nd, 4th, 5th and 9th serial transplantations previously described and tumor material of the same derivation was transferred to both types of animals, but the incidence of takes in tumor bearing animals was always less than in normal animals of the same generation and did not increase with the incidence of takes in normal animals.

The latent period was of similar duration in both types of animals and the subsequent progress of growth in three of the tumor bearing animals was comparable with that observed in normal members of the same generation. One animal was killed on the 60th day and the tumor which occupied approximately 2/3 of the chamber showed the usual histological characteristics noted at that period. In another instance, the growth had replaced the anterior chamber on the 83rd day and had invaded the posterior chamber when the animal was killed on the 141st day. The anterior chamber of the third animal was completely filled on the 52nd day but at autopsy on the 105th day the tumor had almost entirely disappeared and the remaining portion was largely necrotic.

The progress of growth in the remaining two animals differed radically from that noted in normal animals. The growth rate was extremely slow in both instances and the transplants had no more than doubled in size after 130 days. At this time the animals were killed. Microscopic examination showed that the growth in one animal was almost entirely necrotic while the tumor in the other animal was characterized by an abundant myxoid stroma with epithelial cells arranged in well defined acinar formations (Fig. 8), despite the fact that the tumor in normal animals of the same generation showed an almost solid cellular structure with a minimum of supporting elements.

The success of transplantation in these instances appeared to be directly proportional to the size and age of the spontaneous tumors as judged by morphological examination and by a study of the breeding histories of the affected animals. The transfer of fragments to animals with small, early tumors resulted in three failures and two small slow growing nodules, while the inoculation of animals with large, older tumors was followed by takes and rapid growth in all cases.

Other Reinoculation Experiments

A series of experiments was designed with the view of obtaining more information regarding the effect of a growing tumor on the transplantation of other tumors of the same and of diverse nature. The performance of the experiments has been delayed because of the low incidence of takes in the testicle during the summer months, which forced a temporary discontinuance of the series. While relatively few of the experiments have been completed and the available data do not justify conclusions, the results so far obtained seem to be of sufficient interest to warrant recording.

The inoculation of the left testicle with tumor material obtained from the right testicle after its removal from the body has been attempted in two instances. In one, the right testicle was removed 35 days after a successful transfer and the immediate inoculation of the left testicle resulted in a take. The subsequent growth of the nodule in the left testicle was similar in all respects to that previously observed in the right. In the second instance, on the other hand, the affected testicle was not removed until the 135th day and inoculation of the remaining testicle was not followed by growth. Control inoculations resulted in 57.1 per cent of takes in the first instance and in 100 per cent of takes in the second instance.

An attempt to inoculate the testicle with material obtained from biopsy of the eye 66 days after transplantation into that organ was likewise unsuccessful although the inoculated tissue grew in the testicles of all of the control animals.

In another experiment, the tumor was successfully transplanted to the anterior chamber of the right eyes of 13 young animals. The chambers were eventually filled by the growth which subsequently underwent complete regression and left extensive corneal scars. 167 days after the first transplantation, fragments of an actively growing tumor derived from the eye of a member of the 11th serial generation were transferred to the anterior chamber of the left eyes of the recovered animals and 8 controls were inoculated at the same time. Growth resulted in all of the controls but in no instance did a take occur in the reinoculated animals.

Postmortem Examination

A detailed postmortem examination was made of all animals included in these experiments, and in view of the changes found in animals bearing spontaneous uterine tumors, particular attention was directed toward the endocrine system. In the majority of cases, the organs were not pathologically altered and lesions that were found were traceable to disorders common in the general animal population. In no instance were changes comparable to those observed in animals bearing spontaneous uterine tumors found in animals bearing transplanted uterine tumors.

Inoculation with Dried Frozen Tumor Material

A watery emulsion of dried frozen tumor material was inoculated, at laparotomy, into the uterine mucosa of 3 virgin females and 3 multiparae. The animals were killed 42 days after inoculation and the uterine mucosa was serially sectioned. Microscopic examination of these sections and of sections from other organs showed no alteration from normal. Controls inoculated with the living tumor, on the other hand, gave 100 per cent of takes.

DISCUSSION

In the report dealing with spontaneous tumors of the uterus, especial emphasis was placed upon the constant occurrence of certain endocrine changes most evident in the pituitary, suprarenals and thyroid. In brief, the alterations in the pituitary were productive in nature while those in the thyroid and suprarenals were retrogressive. These alterations were present from the earliest stages of tumor development and were also found, before histological evidence of neoplasia, in older animals of the tumor line. This, in itself, suggests the possibility of an antecedent change in the endocrine mechanism which had some bearing on the eventual development of the neoplastic process. This suggested relation is supported by the fact that the conditions observed in animals with spontaneous tumors bear a striking resemblance to those in animals subjected to long continued treatment with estrone, and from this it was inferred that spontaneous tumor development might represent a natural analogue to the experimental production of neoplasia with such substances.

The point to be emphasized in the present discussion is that, despite the fact that the uterine tumor under consideration has been successfully transplanted into more than 150 rabbits and has grown progressively for periods up to 11 months, none of these animals has shown changes at autopsy which bore the slightest resemblance to those constantly present in animals with spontaneous tumors. It is apparent, therefore, that the endocrinological changes are not produced by the continued growth of neoplastic cells. It is also clear that the constitutional status associated with the endocrine changes is not an essential factor in the continued growth of neoplastic cells. In fact, the failure of transplanted tumors to grow actively or to grow at all in animals with early spontaneous tumors suggests that initially there is a contrary or inhibitory influence associated with such changes. It would appear then that the endocrinological conditions referred to were concerned with the initiation of neoplasia and that in the study of this tumor there are, as usual, two distinct problems to be considered, namely: the initiation of neoplasia and the continued multiplication and growth of neoplastic cells.

Investigation of the first problem is being carried out from the point of view of an exogenous as well as of an endogenous origin. Experiments based on the suggestion that the endocrine changes were associated with the initiation of neoplasia have been most encouraging and will be reported at a later date. On the other hand, attempts to demonstrate a causative agent of the nature of a filterable virus have so far been unsuccessful. Neoplasia was not initiated by the introduction of dried frozen material into the uterine mucosa. It is obvious, however, that numerous experiments of this character are necessary before a conclusion is warranted and such experiments are being continued.

The growth characteristics of the tumor have been studied in the eye and in the testicle. Both of these sites have been used by other workers in the investigation of other tumors. The testicle came into prominence following the work of Brown and Pearce with the transplantable epithelioma. They also used the anterior chamber in early experiments and reported a uniform series of takes with rapid growth to large tumors which, however, did not metastasize during the period of observation. A single case of spontaneous regression was noted (4). A sarcoma of the lower jaw had previously been successfully transferred to the anterior chamber by Schultze (5) and Happe (6). Their general results were in agreement with those of the present study but a further investigation of the characteristics and potentialities of the chamber as an inoculation site does not appear to have been carried out. The anterior chamber has also been successfully used in homologous transplantation experiments with tumors of other species (7) and was employed in many early heterologous transplantation attempts without apparent success.

While the susceptibility of the anterior chamber to tumor transplantation appears to be well recognized, it has not been widely used as an inoculation site. Certain features associated with its susceptibility and the characteristics of the resulting growth suggest, however, that a more general use might be of advantage. A comparison of the frequency of takes of the uterine tumor in the testicle and in the eye, as in Table III, brings out a number of points of interest. The relatively unimportant reduction in the number of takes in the anterior chamber during the summer months as compared with the complete failure of testicular inoculation suggests that this method of transfer might prove of great value in the maintenance of other tumors which are so frequently lost in testicular and subcutaneous series during this season.

TABLE III

A Comparison of the Percentage of Takes Resulting from Transfer to the Anterior Chamber and to the Testicle throughout the Year

	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Anterior chamber....	100	83.3	55.5	100	76.9	100	75	33.3	100	87.5	87.5
Testicle............	25	62.5	64.7	42.8	0	37.5	25	0	0	50.0	66.6

In the series as a whole, the number of takes resulting from transfer to the anterior chamber was twice as great as followed inoculation into the testicle. This finding is in line with the fact that in no instance were first generation transfers from a spontaneous tumor to the testicle successful and that takes in the testicle were not obtained until the tumor had been passed through three generations of animals by serial eye transfer. While the cellular damage incident to the preparation of an emulsion may have been of influence in the lower frequency of takes in the testicle, the fact that 100 per cent of takes occurred at some transfers without modification of the technique indicates that this was not an important factor. It seems more probable that the greater success in the anterior chamber is related to the slower reaction of the tissues of the eye to the presence of the transplanted fragment. The fragment grows for a longer period of time in the manner of a tissue culture and a degree of adaptation results before the occurrence of a foreign body reaction with the intimate contact between the cells of the host and the graft that determines its immediate fate.

Transplantation into the anterior chamber offers the further advantage of continuous visual examination of the graft. The effects of various procedures can be watched and the rate of growth can be measured easily with a pair of calipers. Metastasis may occur earlier and with greater frequency from testicular growths but the continued life of the animal is an asset in certain types of experiment. On the other hand, there are distinct disadvantages associated with ocular transfers. The anterior chamber is a small confined space surrounded by relatively inelastic tissues and the increased pressure which follows rapid growth may lead to necrosis and regression of the tumor. If the cornea is invaded or ruptured, the external extension of the tumor produces an unsightly fungating mass and trauma may lead to severe hemorrhage or infection. The great advantage of this method of transplantation lies in the relative ease with which first generation transfers from a spontaneous tumor can be effected. During the past four years more than 140 spontaneous rabbit tumors representative of 16 different types of growth have been observed in this laboratory. Attempts were made to transplant the different types of growth and until recently the testicular and subcutaneous routes were almost exclusively employed. It is significant that while these methods of inoculation failed in every instance, four out of five attempts to transfer by means of the anterior chamber proved successful.

It is not known whether the lowered incidence of takes during the summer months, particularly in the testicle, is related to an increased resistance of the animals or a decreased activity of the tumor cells. There is evidence, however, that even in winter months, periods occur during which an animal may be refractory to inoculation and it is conceivable that meteorological conditions more prevalent during the summer may bring about a refractory phase of widespread occurrence.

The spontaneous and the transplanted tumors show many comparable characteristics. In both abnormal and normal hosts, the tumors progress slowly, growth is at first expansive and later infiltrative, and metastasis is a late occurrence. One characteristic of especial interest is shared by the transplanted tumor and by metastases of the spontaneous tumor. In both, there is evidence of an ability of the neoplastic cells to respond to different environmental conditions with an alteration in the degree of differentiation. Thus, in certain situations, metastatic cells grow in a well formed acinar arrangement and appear to be further differentiated than the cells of the primary growth. In like manner, the transplantation of a cellular, poorly differentiated tumor into animals of a special genetic constitution is followed by more highly differentiated growth with the formation of more or less typical structures.

In other respects the transplanted tumor behaves in a different manner and it should be emphasized that the behavior of neoplastic cells in normal animals is not a reflection of their behavior in the primary host where their activity may be influenced by an altered endocrinological status as well as by the presence of other growing neoplastic cells.

The observation has been made repeatedly by workers with some other tumors that, following successful transplantation, a phase occurs during which the animals are refractory to further inoculation of the same growth (8). In the present instance, it was also found that reinoculation gave negative results after continued growth of the transplanted tumor. Further conclusions cannot be drawn from the results obtained to date, but certain findings in regard to the refractory phase are of interest from the point of view of discussion.

Despite opinions to the contrary, it appears to be fairly clear that the refractory phase is brought about by the continued presence and growth of neoplastic cells, rather than by the absorption of products resulting from regressive changes in the tumor. The duration of the refractory phase in experimental animals in which the tumor under discussion had completely regressed was demonstrated to exceed 5 months. On the other hand, metastasis has occurred in other animals 3 months after transplantation. There is some evidence, therefore, that while growing neoplastic cells may bring about a refractory period, their continued presence and growth in the body may shorten the duration of this period.

The presence of a similar refractory phase in animals with spontaneous growths has not been satisfactorily demonstrated. It is generally believed that grafts of a spontaneous tumor are more apt to be successful if placed in another region of the same animal than if transferred to a normal animal and, in early experiments with the present tumor, it was found that subcutaneous autografts almost invariably grew while subcutaneous transfers to normal animals were uniformly unsuccessful. In these experiments, the growth had been present in the spontaneous host for more than a year before autoinoculation was attempted and it is apparent that at this period of tumor development the animals were not refractory.

A determination of the susceptibility of animals in earlier stages of tumor growth to the transplantation of a malignant tumor of the same nature is a more difficult problem owing to the necessity of assembling a sufficient number of suitable animals as well as of determining the age of the spontaneous tumors. It should also be emphasized that in this type of experiment, in contrast to the experiments cited above, autogenous tumor material cannot be used inasmuch as in the early stages it represents benign rather than malignant neoplasia.

The occurrence of a considerable number of spontaneous uterine growths, the age of which could be determined with fair accuracy, offered a unique opportunity for study of this problem, and tumor material derived from various eye generations was transferred to the eyes of animals bearing spontaneous growths in different stages of development. A series large enough to provide significant results has not been tested and the experiment is being continued as more tumor bearing animals become available. The results are not conclusive for this reason but, while it must be borne in mind that further tests may give rise to contradictory findings, the present trend is of sufficient interest to warrant some consideration.

The uniformity of takes in animals with old tumors is in agreement with expectations based on experiments with autografts. On the other hand, the complete failures and the small slow growing nodules arising from transfers to animals with early spontaneous tumors are more in line with the results obtained from the reinoculation of animals bearing transplanted tumors and may indicate the existence of a similar refractory phase. Additional evidence suggesting the presence of a growth inhibiting influence at this period is given by the fact that while neoplastic cells are present in the blood stream at early stages of tumor development, they fail to gain a foothold and grow until late in its course.

SUMMARY

The behavior of a transplanted adenocarcinoma of the uterus of a rabbit has been studied through 12 serial generations in the anterior chamber of the eye and 6 serial generations in the testicle. The transplanted tumor is characterized by slow growth which is at first expansive and later invasive, by an ability to form more or less differentiated structures in response to different environmental conditions and by late metastasis. The endocrinological changes that distinguish animals bearing the spontaneous tumor do not occur in animals bearing the transplanted tumor.

Various experiments were undertaken in an attempt to discover the nature of the factors determining the characteristics of the spontaneous and of the transplanted tumor. It was found that successful transplantation was followed by a phase during which animals were refractory to reinoculation. The results of transplantation into the eyes of animals with spontaneous tumors suggested the existence of a similar phase during the early development of the tumor but the number of observations was not sufficiently numerous to warrant definite conclusions.

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EXPLANATION OF PLATES

Hematoxylin and eosin was the stain employed throughout.

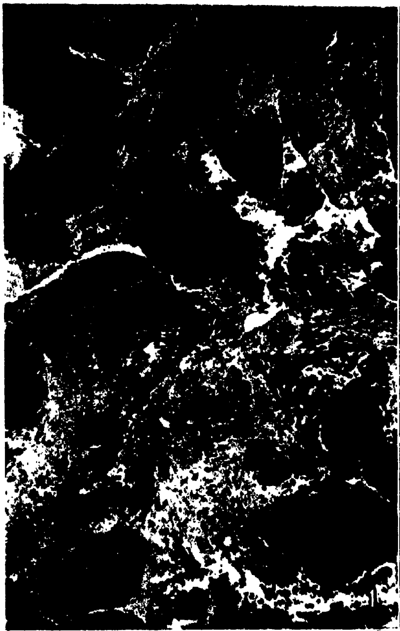
PLATE 28

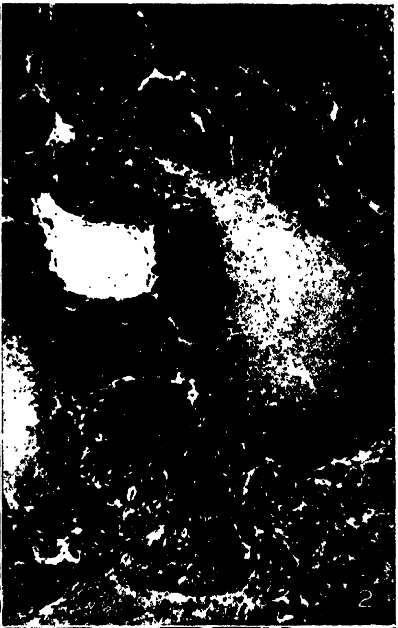
FIG. 1. Section of a transplant in the anterior chamber of the eye resulting from the 2nd serial transfer. Stroma is abundant and epithelial elements tend to grow in abortive acinar formations. × 67.

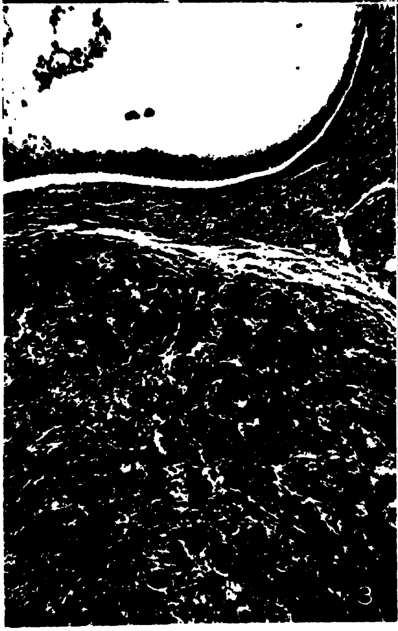
FIG. 2. Section of a transplant in the anterior chamber resulting from the 6th serial eye transfer. In contrast to the previous figure, stroma is sparse and epithelial cells are arranged in large rounded masses which frequently show necrotic centers. × 67.

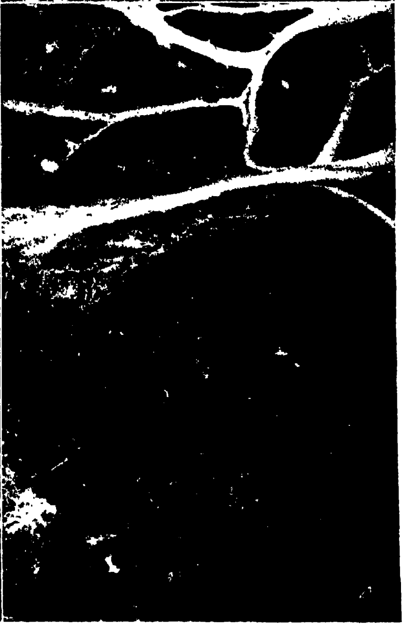
FIG. 3. Section of a metastatic growth in the ovary derived from a transplant in the anterior chamber. × 67.

FIG. 4. Section of a metastatic growth in the pancreas derived from a transplant in the anterior chamber. × 40.5.









Photographed by J. A. Carlile

(Greene: Uterine adenomata in the rabbit. II)

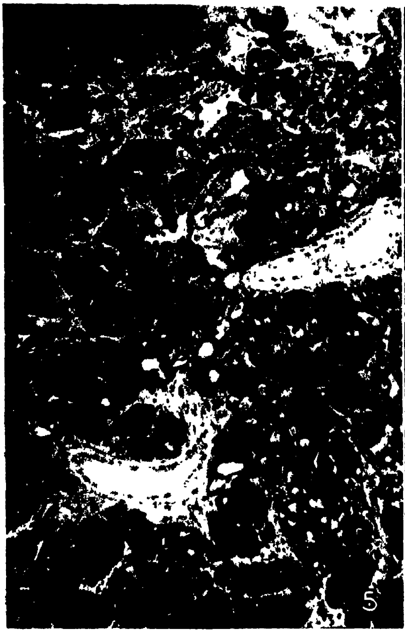
PLATE 29

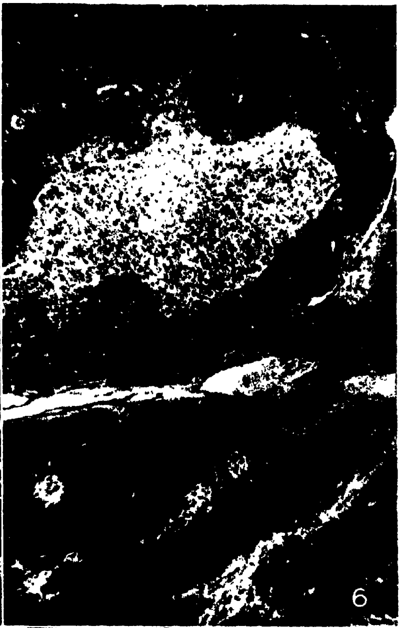
FIG. 5. Section of a transplanted tumor in the testicle of a Himalayan rabbit. Stroma is abundant and epithelial cells grow in acinar-like groups. × 67.

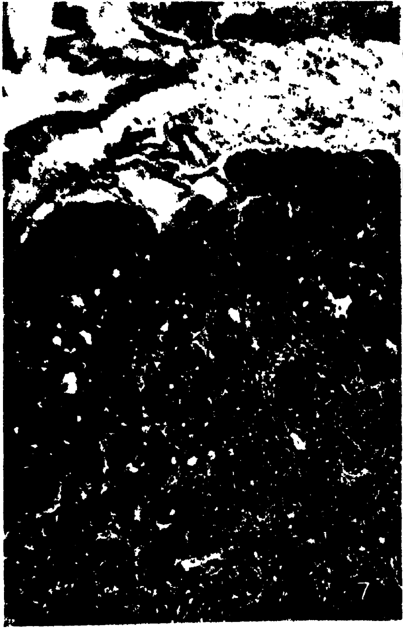
FIG. 6. Section of a transplant in the testicle of a hybrid rabbit showing the characteristic histological features found in the majority of testicular grafts. In contrast to the appearance of the tumor in Himalayan animals, stroma is scanty and the arrangement of epithelial cells is similar to that found in rapidly growing eye transplants. × 67.

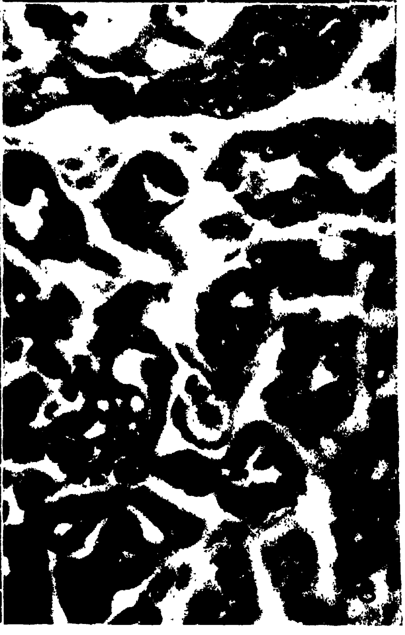
FIG. 7. Section of a metastatic growth in the lung derived from a testicular transplant. × 67.

FIG. 8. Section of a transplant in the eye of a rabbit bearing an early spontaneous uterine tumor of the same nature. Stroma is abundant and epithelial cells are arranged in well defined acinar formations. The appearance of control transplants in normal animals is shown in Fig. 2. × 375.









Photographed by J. A. Carlile

(Greene: Uterine adenomata in the rabbit. II)

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ACQUIRED IMMUNITY TO TICKS

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It is well known that the American dog tick, *Dermacentor variabilis* Say, can be reared through its entire life cycle on guinea pigs. In the course of permitting successive batches of larvae of this tick to engorge on the same guinea pig, it was repeatedly observed that, while the first batch always gave a large number of engorged larvae, the following batches gave few or none. This suggested that guinea pigs, after one infestation with larvae of *D. variabilis*, acquire an effective immunity against these ectoparasites. The experiments reported in this paper show that such an immunity is indeed developed, and that it may be solid enough to prevent larvae from engorging and to reduce the amount of blood taken by nymphs and adults.

Methods

Guinea pigs were used for most of the experiments, rabbits and deer mice (*Peromyscus leucopus*) for a few. In order to permit repeated observation and careful control of the engorging ticks the pill box method (Kohls, 1937) has been employed in all the experiments except those concerned with deer mice. One and one-half or one and one-quarter-inch pill boxes with the bottoms cut out were attached to the previously clipped sides and over the ears of guinea pigs by means of adhesive tape (Plate I, Fig. 1). Rabbits were treated in the same manner except that the ears were fitted with tall cylindrical boxes with a hole cut in the bottom just large enough for the ear to pass through. Counted numbers of ticks were placed in the boxes and observed at intervals during the course of engorgement. Engorged ticks which had dropped off could be readily recovered from the boxes and counted. Occasionally, an animal would succeed in scratching off its ear box. In order to prevent the escape of ticks freed in this manner, all animals with ticks on them were kept in cages made out of one-half-inch mesh wire screen and completely surrounded by a cloth bag tied in front (Kohls, 1937).

The deer mice were trapped in woods near Plainsboro, N. J., during the latter part of March and hence before they could have been infested by larvae or nymphs of *D. variabilis*. They were kept individually in glass battery jars with shavings, and were fed oats, Purina dog chow and water. In order to infest a mouse, a counted number of larvae was sprinkled over it while it was held in a large white pan. All the ticks which fell into the pan were gathered up on a piece of cotton which was put in the jar together with the mouse. The mouse would nest in the cotton, increasing its chances of infestation. Each jar was provided with vaseline at the rim and a wire screen cover, and was held in a large white pan containing water, which caught the few ticks able to get past the vaseline. Two days after the application of the ticks, each mouse was transferred to a fresh jar. Hot water was run into the used jar. Two days still later, each mouse was again placed in a fresh jar. This time, the contents of the old jar were carefully examined for engorged larvae. This process was repeated every other day until all the larvae had dropped from the mouse (usually the eighth day after their application).

The general plan of the experiments with guinea pigs and rabbits has been to use litter mates, usually of the same sex and color, to immunize one and then, after a suitable interval, to apply to both animals ticks descended from the same female and kept under identical conditions. In some experiments litter mates were not used, but a suitable untreated control was always infested at the same time as the animal being tested for immunity, and with ticks from the same batch. Similarly, in each series of infestations of deer mice, larvae from the same batch were used. It is believed that in this manner the great variability in infesting power of different batches of ticks and of the same batch at different times was sufficiently controlled.

RESULTS

I. *Immunity to Larvae.* Table 1 shows that after one infestation of either a guinea pig or rabbit with larvae or nymphs of *D. variabilis* the number of larvae which can subsequently engorge on the animal

TABLE 1

Acquired Immunity to Larvae of Dermacentor variabilis

Exper. No.	Animal	Previous treatment	Region of body*	Ticks put on		No. engorged	Remarks†
				No.	Date		
26	GP 46 ♀	None	L.S.	100	3/ 9/38	18	
			R.S.	100	"	14	
			L.E.	100	"	64	
			R.E.	100	"	77	
		1 infestation started on 3/9/38	L.E.	100	3/15/38	1	Small and browhish
	GP 47 ♀ Litter mate of 46	Wore boxes only for same period as 46	L.E.	100	"	33	
28	Rabbit 1 ♂	None	L.S.	100	3/11/38	6	
			R.S.	100	"	29	
		1 infestation started on 3/11/38	L.S.	114	3/25/38	0	
			R.S.	86	"	0	
	Rabbit 2 ♂ Litter mate of 1	Wore boxes only from 3/11 to 3/18/38	L.S.	86	"	10	
			R.S.	114	"	12	
15	GP 29 ♀	1 infestation with nymphs, 1/13 to 1/23/38	L.S.	50	2/ 1/38	0	
	GP 30 ♀ Litter mate of 29	Wore boxes only from 1/13 to 1/23/38	L.S.	50	"	8	
15a	GP 29 ♀	2 infestations with nymphs on sides of body, last on 2/1 to 2/11/38	L.S.	100	2/24/38	0	
			R.S.	100	"	1	Light brown
	GP 41 ♀	None	L.S.	100	"	29	
			R.S.	100	"	34	
15b	GP 29 ♀	As in 15a. Immune to larvae on sides	L.E.	100	3/ 3/38	0	
	GP 41 ♀	1 infestation with larvae just ending	L.E.	100	"	11	Smaller than normal
16	GP 31 ♀	None	L.E.	100	1/13/38	20	
		1 infestation on left ear, 1/13 to 1/18/38	L.E.	100	1/20/38	4	All 7 engorged larvae of a light brown color
			R.E.	100	"	3	

* L.S., left side; R.S., right side; L.E., left ear; R.E., right ear.

† Remarks omitted when the engorged larvae were of normal size and color.

TABLE 1—*Continued*

Exper. No.	Animal	Previous treatment	Region of body*	Ticks put on		No. engorged	Remarks†
				No.	Date		
	GP 32 ♀ Litter mate of 31	Box only on left ear 1/13 to 1/18/38	L.E.	100	"	51	
	GP 31	1 infestation on left ear, almost immune on both ears on 1/20	L.S.	50	1/25/38	0	
			L.E.	50	"	0	Dead attached unfed larvae noted on both ears
			R.E.	50	"	0	
	GP 32	1 infestation on left ear, box on right ear 1/20 to 1/24/38	L.S.	50	"	0	
			L.E.	50	"	2	One of the 3 engorged larvae was light red, the other 2 undersized and dark brown
			R.E.	50	"	1	
17	GP 33 ♂	None	L.E.	100	1/18/38	15	
		1 infestation on left ear 1/18 to 1/25/38	L.E.	100	1/25/38	0	Several attached but dead larvae noted
			R.E.	100	"	10	
	GP 34 ♂ Litter mate of 33	Box only on left ear 1/18 to 1/25/38	L.E.	100	"	30	
22	GP 12 ♂	1 infestation around center of body on 11/16 to 11/23/37. Immune on 12/1/37	L.S.	100	2/14/38	0	
			L.E.	100	"	2	Both engorged larvae brown
	GP 39 ♀	None	L.S.	100	"	13	
			L.E.	100	"	26	
		1 4-day-old infestation	R.S.	100	2/18/38	25	
			R.E.	100	"	30	
	GP 40 ♀ Litter mate of 39	Boxes on sides and left ear starting on 2/14/38	R.S.	100	"	49	
			L.E.	100	"	58	
		1 infestation started on 2/18/38	L.S.	100	2/23/38	0	
			R.E.	100	"	35	A few of these were dark brown rather than black

* L.S., left side; R.S., right side; L.E., left ear; R.E., right ear.

† Remarks omitted when the engorged larvae were of normal size and color.

TABLE 1—*Continued*

Exper. No.	Animal	Previous treatment	Region of body*	Ticks put on		No. engorged	Remarks†
				No.	Date		
35	GP 46 ♀	1 infestation with larvae on both sides and both ears. 1 infestation with nymphs 3/15 to 3/25/38	L.S.	100	4/ 6/38	0	
			L.E.	100	"	3	7 originally seen attached. Of the 3 engorged, 1 was reddish brown
	GP 47 ♀ Litter mate of 46	1 infestation with nymphs and larvae on sides and ears 3/15 to 3/25/38	L.S.	100	"	0	
			L.E.	100	"	17	3 normal, 6 undersized, 6 undersized and brown, 2 very pale
	GP 30 ♀	1 infestation with larvae and nymphs on left side only 2/1 to 2/11/38	L.S.	100	"	0	
			L.E.	100	"	41	Several of these very pale
	GP 60 ♀	None	L.S.	100	"	12	
			L.E.	100	"	58	
35a	GP 46 ♀	1 infestation with larvae. 2 with nymphs. Last on 4/6 to 4/16/38	L.E.	100	4/26/38	3	1 of these undersized
	GP 47 ♀	1 infestation with nymphs and larvae, 1 with nymphs on 4/6 to 4/16/38	L.E.	100	"	2	8 originally seen attached. Of the 2 engorged, 1 was brown, other undersized
	GP 30 ♀	2 infestations with larvae and nymphs, last on 4/6 to 4/16/38, when partly immune to larvae	R.E.	100	"	11	4 undersized, 1 light red, 6 very pale. Large blister at site of attachment of a cluster of ticks most of which died when only slightly engorged
	GP 62 ♀	None	R.E.	100	"	72	

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* L.S., left side; R.S., right side; L.E., left ear; R.E., right ear.

† Remarks omitted when the engorged larvae were of normal size and color.

TABLE 1—*Concluded*

Exper. No.	Animal	Previous treatment	Region of body*	Ticks put on		No. engorged	Remarks†
				No.	Date		
36	GP 32 ♀	1 infestation on left ear only. Immune on 1/25/38	R.S.	100	4/21/38	2	
			L.E.	100	"	36	A few were brown or undersized
	GP 61 ♀	None	R.S.	100	"	62	
			L.E.	100	"	99	
42	GP 36 ♀	1 small infestation with larvae on left side only, 2/1 to 2/7/38	L.S.	100	5/19/38	0	
			L.E.	100	"	5	Undersized. Some others attached but died without feeding
			R.E.	100	"	5	
	GP 50 ♀	Nymphs on right side, ear, larvae on left side, ear from 3/25 to 4/4/38	L.S.	100	"	0	
			L.E.	100	"	0	Few attached on both ears but failed to feed
			R.E.	100	"	0	
	GP 55 ♂	Nymphs on both sides, larvae on left ear, 4/16 to 4/26/38	L.S.	100	"	0	
			L.E.	100	"	0	About 16 attached but dropped off without engorging
			R.E.	100	"	3	Very small. Small blisters on ear
	GP 61 ♀	1 infestation with larvae on right side and left ear 4/21 to 4/27/38	L.S.	100	"	0	
			L.E.	100	"	1	Several others attached but dropped off without feeding
			R.E.	100	"	5	Undersized. Blisters on both ears
	GP 68 ♀	None	L.S.	100	"	28	
			L.E.	100	"	65	
			R.E.	100	"	57	

* L.S., left side; R.S., right side; L.E., left ear; R.E., right ear.

† Remarks omitted when the engorged larvae were of normal size and color.

is greatly reduced. Exper. 15b indicates that the immunity is not a strictly local one. Guinea pig (hereafter abbreviated in text and tables as GP) 29, which had had two infestations with nymphs applied only on the sides of its body, completely resisted the attack of larvae placed on its ear. Tested similarly, GP 41 was partially immune: after one infestation with larvae on its sides only started 7 days previously, 11 out of 100 larvae engorged, but were smaller than normal.

Exper. 16 gives further data on the development and spread of the immunity. The engorgement of only 20 larvae on the left ear of GP 31 rendered both ears partially immune one week later, and both ears and the left side completely immune 12 days after the start of the first infestation. GP 32, litter mate of GP 31, which wore only a box on its left ear during the first infestation of GP 31, was highly susceptible, 51 out of 100 larvae engorging in a normal manner. Exper. 17 shows again the spread of the immunity from the left to the right ear. The first part of Exper. 22 shows that one infestation on the sides of the body rendered the ear as well as the sides immune. In the case of GP 39, the right side and right ear, 4 days after the start of infestations on the left side and left ear, already showed some immunity as compared with the litter mate GP 40.

The result with GP 12 in Exper. 22 indicates that the immunity lasts at least 3 months. This is borne out by the data in Exper. 35, 36, and 42. These experiments also show clearly that the immunity is more effective on the sides of the body than on the ears. Even on previously untreated animals, however, usually a higher percentage of larvae was able to engorge on the ears than on the sides. GP 30 (Exper. 35), infested on its left side early in February, was still completely immune on the left side 2 months later, but only partially immune on its left ear. Forty-one larvae, some of them very pale in color, engorged on the ear of GP 30 as compared with 58 normal black larvae on the ear of control GP 60. However, when larvae were applied to the right ear of GP 30 (Exper. 35a) 10 days after the end of a fourth infestation with nymphs, running concurrently with the larval infestation on its left ear, only 11 engorged, none of them normal, as compared with 72 normal engorged larvae on the right ear of control GP 62.

In Exper. 36, the data on GP 32 show that this animal retained a partial immunity for 3 months. GP 36 (Exper. 42), which had one small infestation early in February on its left side only, showed, in the middle of May, almost as much immunity on its sides and ears as GP 50, 55 and 61, last infested in April. The results of Exper. 42 are perhaps especially striking in that they show the very great difference in number of engorged larvae obtained from a previously uninfested animal (GP 68) and from four different animals having one thing in common, namely, that they had been previously infested at least once with larvae or nymphs of *D. variabilis*.

TABLE 2

Cross-Immunization between Larvae of Dermacentor variabilis and Haemaphysalis leporis palustris

Exper. No.	Animal	Previous treatment	Region of body*	No. larvae put on of		Date put on	No. engorged
				H. leporis palustris	*D. variabilis*		
39	Rabbit 3	None	L.S.	0	300	5/ 9/38	16
		1 infestation just ending	R.E.	0	200	5/17/38	44
		2 successive infestations with *D. variabilis*	L.S.	100	0	5/26/38	1
			R.S.	0	100	"	0
			L.E.	0	100	"	3
			R.E.	100	0	"	1
	Rabbit 4 Litter mate of 3	Boxes only, at same time as Rabbit 3	L.S.	100	0	"	8
			R.S.	0	100	"	1
			L.E.	0	100	"	72
			R.E.	100	0	"	78
	Rabbit 5	None	L.S.	300	0	5/ 9/38	42
		1 infestation just ending	R.E.	200	0	5/17/38	12†
		2 successive infestations with *H. leporis palustris*	L.S.	0	100	5/25/38	13
			R.S.	100	0	"	5
	Rabbit 6 Litter mate of 5	Boxes only, at same time as Rabbit 5	L.S.	0	100	"	26
			R.S.	100	0	"	23

* L.S., left side; R.S., right side; L.E., left ear; R.E., right ear.

† Got ear box off on first day.

A few experiments with larvae of *D. variabilis*, *Haemaphysalis* *leporis palustris* Packard[1] and *Dermacentor andersoni* Stiles[2] have shown that each of these species induces some immunity to itself and to larvae of the other species. Table 2 shows the cross immunity between *D. variabilis* and *H. leporis palustris.* Two litter mate pairs of rabbits were used. One rabbit of one pair was infested with *D. variabilis*, one rabbit of the other pair with *H. leporis palustris.* Subsequently, both species of ticks were placed on both rabbits of each pair. In each case, the previously infested rabbit showed a high degree of immunity to both species.

Table 3 shows the cross immunity between *D. variabilis* and *D. andersoni.* Of three litter mate guinea pigs, one was infested with *D. variabilis* and a second with *D. andersoni.* All three were then infested with both species and again the previously infested animals showed partial immunity to both. The animal infested with *D. variabilis* on its left side and left ear subsequently gave 11, 11 and 19 engorged *D. andersoni* on its left side, left ear and right ear respectively, as compared with 64 and 22 on the left side and left ear of the control. GP 78 underwent on its right side and right ear only a very light original infestation with *D. andersoni* (the larvae used for this infestation, unlike those used for the test infestations, were from a weak batch found to have difficulty in engorging even on previously uninfested animals). Nevertheless, the test infestation gave only 1, 60 and 2 engorged *D. variabilis* on the animal's right side, left ear and right ear respectively, as compared with 55 and 81 on the right side and right ear of the control. In GP 78 there was only slight spread of the immunity to the previously uninfested left ear.

Neither guinea pigs nor rabbits are natural hosts of larvae of *D. variabilis.* Hence it became important to discover whether a natural host, such as the deer mouse (Parker, Philip, Davis and Cooley, 1937), could develop an immunity to the tick larvae. A number of experiments showed that one infestation with larvae of *D. variabilis* induced no immunity and indeed even appeared to make the animal more susceptible. Two or three infestations did however induce an effective

[1] The larvae of *H. leporis palustris* were descended from a single engorged female obtained from an Oklahoma cottontail rabbit received by Dr. R. E. Shope.

[2] The larvae of *D. andersoni* were descended from females which were sent me in the unfed state through the kindness of Dr. R. R. Parker.

immunity, as shown in table 4. In two separate tests, mice 2 and 3, which had had three and two, and four and three, infestations respectively, supported the engorgement of fewer larvae than did the control mice. It is likely that deer mice never become completely immune. However, the relatively immune animals showed a noticeable difference in appearance from the susceptible animals. Plate I, Fig. 2, is a photograph of deer mouse 2 on the 5th day of its fourth infestation. Plate I, Fig. 3, shows deer mouse 6 on the 5th day of its first infestation. This mouse, before ticks had been applied to it, had a coat of fur even more sleek and smooth than that of mouse 2. Mouse 2, during its first three infestations, and all mice during their first infestations, presented the same appearance as mouse 6. The fur became very wet and ruffled, probably from the excessive washing

TABLE 3

Cross-Immunization between Larvae of Dermacentor variabilis and Dermacentor andersoni

Exper. No.	Animal	Previous treatment	Region of body*	No. larvae put on of		Date put on	No. engorged
				D. andersoni	*D. variabilis*		
46	GP 77 ♀	None	L.S.	0	100	6/ 8/38	31
			L.E.	0	100	"	38
	GP 78 ♂ Litter mate of 77	None	R.S.	100	0	"	10
			R.E.	100	0	"	3
	GP 77	1 infestation with *D. variabilis* from 6/8 to 6/15/38	L.S.	100	0	6/21/38	11
			R.S.	0	100	"	3
			L.E.	100	0	"	11
			R.E.	100	0	"	19
	GP 78	1 light infestation with *D. andersoni* from 6/8 to 6/15/38	L.S.	100	0	"	19
			R.S.	0	100	"	1
			L.E.	0	100	"	60
			R.E.	0	100	"	2
	GP 79 ♂ Litter mate of 77, 78	Wore boxes only, from 6/8 to 6/15/38	L.S.	100	0	"	64
			R.S.	0	100	"	55
			L.E.	100	0	"	22
			R.E.	0	100	"	81

* L.S., left side; R.S., right side; L.E., left ear; R.E., right ear.

given it by the mouse in its attempts to dislodge the ticks. Several days after the larvae had all dropped off, the fur again became normal. In the series of infestations started on June 14, 1938 (Table 4), mouse 2, undergoing its fifth infestation, retained sleek smooth fur. The fur of mouse 3 was rather wet and ruffled, while that of mice 5, 7, and 11 was decidedly so. The fact that mice 3 and 5 showed wet ruffled fur in spite of the much fewer numbers of larvae which engorged on them than on mice 7 and 11 suggests that on the former partially immune animals many more larvae attached and started to engorge than were able to complete engorgement.

TABLE 4

The Development by Deer Mice of Acquired Immunity to Larvae of Dermacentor variabilis

Deer mouse No.	Previous treatment	Ticks put on		No. engorged
		No.	Date	
2	None..................................	300	4/13/38	29
2	1 infestation: 4/13 to 4/20...............	400	4/27/38	80
3	None..................................	400	"	33
2	2 infestations: 4/13 to 4/20 and 4/27 to 5/4..	400	5/14/38	33
3	1 infestation: 4/27 to 5/4...............	400	"	54
5	None..................................	400	"	38
2	3 infestations: 4/13 to 4/20, 4/27 to 5/4 and 5/14 to 5/21.........................	400	5/31/38	10
3	2 infestations: 4/27 to 5/4 and 5/14 to 5/21.	400	"	8
6	None..................................	400	"	69
2	4 infestations: 4/13 to 4/20, 4/27 to 5/4, 5/14 to 5/21 and 5/31 to 6/7...............	400	6/14/38	12
3	3 infestations: 4/27 to 5/4, 5/14 to 5/21 and 5/31 to 6/7.........................	400	"	22
5	1 infestation: 5/14 to 5/21...............	400	"	37
7	None..................................	400	"	185
11	None..................................	400	"	118

II. *Immunity to Nymphs and Adults of* D. variabilis. In these experiments, the nymphs placed on corresponding regions of test and control animals were always descendants of the same female, and, as larvae, had engorged on the same susceptible guinea pig at the same time. Early in the course of the work it was observed that previous infestations with larvae or nymphs had little or no effect on the number of nymphs able to attach and engorge. There seemed to be some effect on the size, however. A series of experiments, some of which are presented in table 5, has given entirely consistent results and shown that the engorged nymphs dropping from previously infested guinea pigs weigh less than the corresponding nymphs from control guinea pigs. The average weight of nymphs engorged on control guinea pigs was always about 10 mg. As may be seen in table 5, there was a fair correlation between the number and type of previous infestations and the reduction in average weight of the engorged nymphs. For example, GP 16, which had had two successive infestations with larvae and was immune to reinfestation with larvae, gave, on its first infestation with nymphs (Exper. 32), engorged ticks of almost normal average weight (9.3 mg as compared with 12.5 mg on the control). When this guinea pig was again infested with nymphs, the average weight of the engorged ticks on the two sides was only 6.7 mg and 7.5 mg (Exper. 32a), as compared with 12.7 mg and 10.7 mg on the control animal. In the same experiment, GP 24 and 29, which had had four infestations with nymphs, gave engorged nymphs which were still smaller than those from GP 16, and also smaller than the nymphs obtained from GP 24 and 29 after only three infestations (Exper. 32). As might be expected, there was considerable variation among individual guinea pigs with respect to the degree of immunity developed. Nymphs engorging on immune guinea pigs produced much less black excreta than did those engorging on control animals, a further indication that the former take up less blood than the latter.

All the engorged nymphs were kept in small cotton-plugged test tubes over moist sand. Practically all the nymphs which had fed on control guinea pigs molted to good sized or even large vigorous adults. Most of the small nymphs engorged on immune animals molted to adults of normal appearance but of small size. Some of these nymphs gave rise to adults only 2 to 3 mm long which lacked the adult pigmentation. Superficially, they resembled overgrown nymphs. They had the same brown color as nymphs and were quite transparent. The genital pore was present, however, and males and females could be distinguished by this and by the relative size of the scutum, which, as in normal ticks, covered the dorsum of the males completely and that of the females only partially. The structures surrounding the

TABLE 5

Acquired Immunity to Nymphs of Dermacentor variabilis

Exper. No.	Animal	Previous treatment	Region of body*	Ticks put on		Engorged ticks	
				No.	Date	No.	Av. wt.
							mg
15	GP 29 ♀	2 infestations with nymphs starting on 1/13 and 2/1/38	L.S.	15	2/24/38	13	3.9
			R.S.	16	"	15	3.7
	GP 41 ♀	None	L.S.	15	"	14	10.1
			R.S.	16	"	15	9.7
32	GP 16 ♂	2 successive infestations with larvae. Immune to larvae on 12/24/37	R.S.	20	3/25/38	18	9.3
	GP 24 ♂	3 infestations with nymphs started on 12/14/37, 1/3 and 1/17/38	R.S.	20	"	20	6.4
	GP 29 ♀	3 infestations with nymphs started on 1/13, 2/1 and 2/24/38	R.S.	20	"	16	6.5
	GP 50 ♀	None	R.S.	20	"	18	12.5
32a	GP 16 ♂	Immune to larvae on 12/24/37. 1 infestation with nymphs started on 3/25/38	L.S.	20	4/16/38	15	6.7
			R.S.	20	"	19	7.5
	GP 24 ♂	4 infestations with nymphs started on 12/14/37, 1/3, 1/17 and 3/25/38	L.S.	20	"	15	4.7
			R.S.	20	"	19	5.8
	GP 29 ♀	4 infestations with nymphs started on 1/13, 2/1, 2/24 and 3/25/38	L.S.	20	"	16	3.5
			R.S.	20	"	2	1.7
	GP 55 ♂	None	L.S.	20	"	18	12.7
			R.S.	20	"	19	10.7
35	GP 46 ♀	1 infestation with larvae, 2 with nymphs started on 3/15 and 4/6/38	L.S.	20	4/26/38	14	5.4
			R.S.	15	"	14	4.4
	GP 47 ♀ Litter mate of 46	1 infestation with nymphs and larvae, 1 with nymphs, started on 3/15 and 4/6/38 respectively	L.S.	20	"	13	6.2
			R.S.	15	"	11	7.2
	GP 30 ♀	1 infestation with nymphs and larvae, 1 with nymphs, started on 2/1 and 4/6/38 respectively	L.S.	20	"	16	2.6
			R.S.	15	"	11	4.6
	GP 62 ♀	None	L.S.	20	"	19	9.7
			R.S.	15	"	6†	11.2

* L.S., left side; R.S., right side.

† Some nymphs escaped on first day, as box was loose.

genital pore were less developed than in normal ticks. No attempt has been made to engorge these abnormal adults, so that nothing is known of their reproductive capabilities.

The immunity of guinea pigs to adult female ticks manifested itself in much the same ways as did their immunity to the nymphs. Females which engorged on animals previously infested with nymphs or adults weighed less and produced less black excreta than control females engorging on previously uninfested animals. Some of the results with adults are given in table 6. Exper. 41 and 44 were done with reared adults, ticks entirely comparable in parentage and treatment being placed on corresponding regions of the test and control guinea pigs. In Exper. 43, wild ticks collected in Princeton were used. It will be noted that on control animals, wild females became larger than did the laboratory-reared females. Exper. 41 shows that larval infestations conferred no immunity to the adults, whereas repeated nymphal infestations, and even more, nymphal plus adult infestations, greatly reduced the average weight of the engorged females and the weight of black excreta per engorged female. Exper. 44 shows the difference between adult females engorged on litter mate guinea pigs both of which had had one larval infestation but only one of which had been previously infested by adults. The ticks on the two sides of the animals came from two different groups, so that comparison can be made only between the ticks on corresponding sides. On each side, the ticks engorged on the animal which had had one previous adult infestation were somewhat smaller and produced less excreta than those engorged on the litter mate control.

In Exper. 43, it may be seen how repeated nymphal infestations reduced the amount of blood taken by adult females. It is worthy of note that GP 29, which showed the highest degree of immunity to nymphs (Table 5, Exper. 32a), also showed the highest degree of immunity to the adults. Repeated nymphal infestations conferred more immunity to adults than one small adult infestation. It is possible that repeated heavy infestations with adults might produce a much more pronounced degree of immunity to the adults than has so far been observed. Jellison and Kohls (1938) placed successive small lots of adult *D. andersoni* on one region of rabbits and noted that toward the close of the experiment newly added ticks were un-

TABLE 6

Acquired Immunity to Adults of Dermacentor variabilis

Exper. No.	Animal	Previous treatment	Region of body*	Date 4 ♀ ticks put on†	Engorged ♀ ticks		Wt. excreta per engorged ♀ tick
					No.	Av. wt.	
						mg	*mg*
41	GP 5 ♂	2 nymphal, 2 adult infestations, last on 3/28 to 4/16/38	L.S.	5/20/38	4	294	37
			R.S.	"	3	249	33
	GP 6 ♂	1 nymphal, 1 adult infestation, last on 3/28 to 4/16/38	L.S.	"	4	175	24
			R.S.	"	4	245	24
	GP 47 ♀	1 larval, 3 nymphal infestations, last on 4/26 to 5/6/38	L.S.	"	4	295	41
			R.S.	"	4	509	62
	GP 20 ♂	2 larval infestations. Immune to larvae on 12/28/37	L.S.	"	4	538	127
	GP 73 ♂	None	L.S.	"	4	556	81
43	GP 24 ♂	5 nymphal infestations, last on 4/16 to 4/26/38	L.S.	6/ 6/38	4	598	100
			R.S.	"	4	744	118
	GP 29 ♀	5 nymphal infestations, last on 4/16 to 4/26/38	L.S.	"	4	395	76
			R.S.	"	3	356	110
	GP 30 ♀	1 infestation with larvae and nymphs, 2 with nymphs. Last on 4/26 to 5/6/38	L.S.	"	4	632	50
			R.S.	"	4	609	92
	GP 73 ♂	1 infestation with adults on 5/20 to 5/30/38	L.S.	"	4	696	137
			R.S.	"	4	721	124
	GP 75 ♂	None	L.S.	"	4	779	158
			R.S.	"	4	818	128
	GP 76 ♂	None	L.S.	"	4	750	133
			R.S.	"	3	797	112
44	GP 20 ♂	Immune to larvae. One adult infestation on 5/20 to 5/30/38	L.S.	"	4	553	103
			R.S.	"	4	346	34
	GP 21 ♂ Litter mate of 20	1 larval infestation	L.S.	"	4	589	131
			R.S.	"	4	446	85

* L.S., left side; R.S., right side.

† An equal number of male ticks was put on with each group.

able to attach on the encrusted area, while those which were attached fed slowly. Both of these phenomena may have been simply the result of the encrustation, or the immunity here reported may also have played a part. It should be emphasized that in the experiments with nymphs and adults detailed in the present paper sufficiently small numbers were used and enough time was allowed to pass between infestations so that, at the time of reinfestation, the scabs formed from the previous infestation were almost or completely healed and covered with new growth of hair. Moreover, in some experiments the immunizing infestation was applied on one side of the body, and the test dose on both sides.

III. *Artificial and Passive Immunization.* In these studies, only the larvae of *D. variabilis* were used, as they furnished the best and most convenient test organism.

Several experiments, two of which are given in table 7, have shown that the acquired immunity of guinea pigs can be artificially produced by the intracutaneous inoculation of an extract of larval ticks. The extract used for Exper. 24 was prepared by grinding approximately 400 larvae in 0.85% sodium chloride solution, centrifuging and diluting the supernatant with the saline solution to 8 cc. That used for Exper. 34 was prepared by grinding about 500 larvae in saline solution, centrifuging and diluting the supernatant to 6 cc. Both extracts were slightly yellowish and opalescent. As is clearly shown in table 7, a series of injections on the left side only with either of these extracts produced a rather solid immunity on both sides and a partial immunity on the ear. The control guinea pigs were inoculated with saline solution, except for GP 59 which received an extract of 7 adult female *Aedes aegypti* mosquitoes in 6 cc of saline solution. As indicated in table 7, guinea pigs receiving tick extract developed small, circular, reddish, hardened, depilated areas at the site of injection.

It has been possible to transfer the immunity passively by the intraperitoneal inoculation of serum from "hyperimmune" animals (Table 8). Guinea pigs which had been repeatedly infested with nymphs, such as GP 24, 29, 30 (Table 5) were considered as having been hyperimmunized and were the serum donors for the passive immunization experiments. Blood was obtained by heart puncture. The first inoculation with serum was always performed immediately after the application of the ticks. As shown in table 8, the immunity conferred by passive transfer was usually not a very solid one, but it consistently

TABLE 7

Artificial Immunization to Larvae of Dermacentor variabilis

Exper. No.	Animal	Treatment	Region of body*	Ticks put on		No. engorged	Remarks
				No.	Date		
24	GP 44 ♀	Injected intracutaneously on left side with larval tick extract—0.05 cc on 3/2/38, 0.1 cc on each of 3/3, 3/4, 3/5 and 3/7	L.S.	100	3/10/38	0	
			R.S.	100	"	1	Brown and undersized
	GP 45 ♀ Litter mate of 44	Injected like 44 but with 0.85% NaCl solution	L.S.	100	"	33	
			R.S.	100	"	25	
34	GP 56 ♂	Injected intracutaneously on left side with 0.1 cc of larval tick extract on 4/2, 4/4, 4/5, 4/6, 4/7/38	L.S.	100	4/14/38	6	On 4/5 shows reddish hardened region at site of injections
			R.S.	100	"	13	On 4/14 only slight scabs remain
			L.E.	100	"	26	1 of engorged larvae brown
	GP 57 ♂ Litter mate of 56	Injected like 56, but with 0.85% NaCl solution	L.S.	100	"	42	No reaction on skin
			R.S.	100	"	41	
			L.E.	100	"	44	
	GP 58 ♂	Injected exactly like 56	L.S.	100	"	0	On 4/14 shows 3 marked red depilated circular areas at site of injection
			R.S.	100	"	1	
			L.E.	100	"	51	
	GP 59 ♂ Litter mate of 58	Injected like 56 but with extract of adult ♀ *Aedes aegypti*	L.S.	100	"	35	No reaction on skin
			R.S.	100	"	11	
			L.E.	100	"	72	

* L.S., left side; R.S., right side; L.E., left ear.

reduced the number of larvae able to engorge by about 50%. In Exper. 40, one inoculation of 1 cc of serum per 100 gm body weight produced as much immunity as did two inoculations of 1 cc or of 1.25

cc. Some of the larvae which engorged on guinea pigs receiving immune serum were pale brown rather than black. Such pale brown larvae were frequently obtained from previously infested hosts (see remarks, Table 1, and section IV of Results), but out of the hundreds of larvae engorged on control guinea pigs all were black except one. These facts furnish further evidence for the transfer of an antibody in the immune serums. The time relation between the application of larvae and the inoculation of serum eliminates the possibility of the immunization having been the result of a transfer of circulating antigen, as in the experiments of Stumberg (1933) on the nematode *Haemonchus contortus*. Moreover, when the donor guinea pigs were bled no ticks were attached to them, so that it was very unlikely that tick antigen was still present in the blood.

A number of attempts to demonstrate antibodies *in vitro* in serums successfully used for passive immunization, failed. Extracts of tick larvae in 0.85% sodium chloride solution were used as antigens. In one case a very faint ring precipitin test was obtained. The complement fixation test could not be used because it was found that normal inactivated guinea pig serum, at dilutions up to 1:32, reacted with the tick extract to fix complement. The tick extract at the concentration used had no anticomplementary or hemolytic action. Neither the normal nor the immune guinea pig serum at the dilutions of 1:8 and higher had any anticomplementary effect. But normal serum plus tick extract fixed complement as much as did immune serum plus tick extract. Preliminary work indicates that rabbit serum will not show such a nonspecific complement fixation with tick extract, in which case it may be possible to demonstrate specific complement fixation with serum from immune rabbits.

IV. *The Cellular Reaction in Non-Immune and Immune Guinea Pigs.* The bites of adult ticks generally produce edematous inflammatory lesions (Hooker, Bishopp and Wood, 1912). Wolbach (1919) has described the histology of the adult tick bite. The epidermis at the point of attachment is absent and the surface of the exposed corium is necrotic and infiltrated with fibrin and leucocytes. The corium is edematous, and new fibroblasts and mononuclear phagocytic cells are present in large numbers. Gregson (1937) has recently studied the histology of adult tick bites on sheep and has also observed an acute edematous

TABLE 8

The Passive Transfer of Immunity to Larvae of Dermacentor variabilis

Exper. No.	Animal	Treatment	Region of body*	Ticks put on		No. engorged	Remarks
				No.	Date		
23	GP 42 ♀	Inoculated intraperitoneally with serum from immune guinea pigs, receiving per 100 gm wt. 0.4 cc on 3/1, 0.8 cc on 3/2, 0.9 cc on 3/3 and 1.1 cc on 3/4/38	L.S.	100	3/ 1/38	2	
			R.S.	100	"	1	This one of a light brown color
	GP 43 ♀ Litter mate of 42	None	L.S.	100	"	30	
			R.S.	100	"	10	
31	GP 53 ♀	Inoculated intraperitoneally with serum from immune guinea pigs, receiving per 100 gm wt. 1 cc on 3/17 and 1.5 cc on 3/18, 3/19, 3/20/38	L.S.	100	3/17/38	14	
			R.S.	100	"	25	
			L.E.	100	"	7	Total = 46
	GP 54 ♀ Litter mate of 53	Inoculated like 53 but with serum from normal guinea pigs	L.S.	100	"	18	
			R.S.	100	"	42	
			L.E.	100	"	26	Total = 86
40	GP 69 ♂	Inoculated intraperitoneally with immune GP serum, receiving per 100 gm wt. 1 cc on 5/12/38	L.S.	100	5/12/38	13	
			R.S.	100	"	8	
			L.E.	100	"	66	Total = 87
	GP 70 ♂ Litter mate of 69	Inoculated intraperitoneally with immune GP serum, receiving per 100 gm wt. 1 cc on 5/12 and on 5/14/38	L.S.	100	"	15	Total = 92. One from ear was pale brown in color
			R.S.	100	"	5	
			L.E.	100	"	72	
	GP 71 ♂ Litter mate of 69	Inoculated intraperitoneally with immune GP serum, receiving per 100 gm wt. 1.25 cc on 5/12 and on 5/14/38	L.S.	100	"	20	Total = 88. About half of those from ear were pale brown in color
			R.S.	100	"	15	
			L.E.	100	"	53	
	GP 72 ♂ Litter mate of 69	Inoculated like 71 but with normal GP serum	L.S.	100	"	46	
			R.S.	100	"	41	
			L.E.	100	"	76	Total = 163

* L.S., left side; R.S., right side; L.E., left ear.

inflammation at the site of attachment. Talice (1930) has described the appearance of sections through the points of attachment of larvae of *Dermacentor reticulatus* on the ear of a hedgehog. His photomicrographs show some thickening of the cornified layer of skin and some inflammatory reaction with a hemorrhagic region at its center. The epithelium is lacking at the site of attachment and is not thickened at the edges of the bite.

In an attempt to discover some visible reaction which could account, at least in part, for the acquired immunity of guinea pigs to infestation by larvae of *D. variabilis*, a study was made of the histology of larval tick bites on the ears of susceptible and partially immune animals. On the 4th to 6th day after the application of the ticks, a small portion of ear bearing attached larvae was cut off and fixed in Carnoy-Lebrun. In order to improve the quality of the sections through the chitinous parts of the ticks, a method described by Slifer and King (1933) for grasshopper eggs was used. After fixation and treatment with iodine in 70% alcohol, the tissues were left one day in 80% alcohol containing 4% phenol. They were then passed through 95% alcohol, aniline oil, xylene, and embedded in paraffin in the usual manner. The paraffin blocks were cut enough to expose the tissue and were then soaked overnight in water. Serial sections of entire tick larvae, nymphs and even adult, recently molted females could be obtained in this way. The sections of the ears and attached larvae were stained by Wolbach's (1919) Giemsa method. Plate II, Fig. 1, is typical of the appearance of sections through the point of attachment of a tick larva on a susceptible guinea pig. This ear was fixed on the 4th day after the application of the ticks, when the attached larvae were almost fully engorged. The epithelium is lacking at the point of attachment. The mouthparts are surrounded by a mass of pink-staining material, presumably fibrin. Beneath them there is generally a small hemorrhagic area but practically no cellular reaction. The epithelium is not thickened and there is no leucocytic infiltration. Plate II, Fig. 2, shows a section through the right ear, fixed on the 4th day after application of the ticks, of a guinea pig previously infested on its left ear only (see Table 1, Exper. 17, GP 33). On the first day after application, numerous attached larvae were noted on this ear, but by the 4th day there were relatively few and these were only partly engorged. A study of a series of sections through the still infested piece of ear has shown reactions of which that pictured in the photomicrograph is entirely typical. The pink-stained mass of fibrin is surrounded by a large inflammatory reaction. The solid mass of leucocytes contains all types of cells, but polymorphonuclears and small polyblasts are especially numerous. There are very few eosinophiles. The epithelium at the edges of the bite is thickened and has grown down beneath the leucocytic mass. The whole region is edematous. Thus, by the 4th day, before the tick larva has been able to engorge, it is effectively walled off from its source of supply of food. This same type of reaction has been seen in sections made from a number of partially immune guinea pigs studied in this manner. Frequently, the reaction is macroscopically visible as a white blister. The intensity of the reaction varies considerably, especially with respect to the degree of epithelial growth. This variation may be due to variations among the ticks as well as among the guinea pigs. On one ear of a partially immune guinea pig two ticks were attached side by side. Beneath the mouthparts of one there was a relatively small leucocytic mass and no epithelial thickening. Beneath the mouthparts of the other there was a large leucocytic mass and the epithelium was thickened and had started to grow down. The former tick had a normal black color at the time the ear was fixed, while the latter tick was a very pale brown.

Attention has already been drawn to the abnormally colored tick larvae so frequently obtained from partially immune hosts. Plate I, Fig. 4, shows a normal, black, engorged larva and one of the very pale brown, almost white larvae. The color of these pale larvae is undoubtedly the result of the cellular reactions just described. Such larvae suck up leucocytes instead of red blood cells. This fact has been confirmed in sections of normal and white engorged larvae. The alimentary tract cells of normal larvae appear very large and full of irregular deep-pink-stained globules. In the lumen of the gut there is a solid dark-rose-colored mass with a few intact red cells and a few leucocytes. There are also many brown granules resulting from the digestion of hemoglobin. In an exactly comparable pale individual, the alimentary tract cells appear small and some distance away from the hypodermis. The lumen of the gut is filled with a diffuse granular light blue staining material containing many leucocytes. Some of the pale engorged larvae successfully molted to decidedly undersized nymphs, but many of them died, although kept under the same conditions as normal engorged larvae, almost all of which molted.

In addition to the very pale larvae and the larvae of various shades of brown, representing gradations between the former and normal larvae, there were occasionally obtained, from immune guinea pigs only, bright red engorged larvae and reddish brown ones. The bright red ones always died soon after detaching. At a stage when the gut contents of normal larvae, examined in the fresh state, contained some large hemoglobin crystals and many black granules, the gut contents of the red larvae contained intact red blood cells and no black granules. It would seem that something had interfered with the normal digestive processes of the tick.

Only one experiment concerned with the cellular reaction to nymphal tick bites has been performed. Two litter mate female guinea pigs were used, one of which was infested with larvae of *D. variabilis* on both sides and both ears. Six days later, 12 nymphs of *D. variabilis* were placed on the right ear of each. Eight days still later the last nymphs to engorge had dropped from both animals. Nine engorged nymphs were obtained from the previously infested one and 12 from the litter mate. The former ticks averaged slightly less in weight than the latter. The right ear of each animal showed small white blisters at the points where the ticks had been attached. Two such regions from the ear of each animal were fixed and sectioned. Sections of both blisters from the previously uninfested animal appeared as shown in Plate III, Fig. 1. The mass of fibrin is continuous with a considerable hemorrhagic region, the epithelium is somewhat thickened, and there is a pronounced inflammatory edema. The picture is very similar to that described by others for adult tick bites. Plate III, Fig. 2, shows the appearance of sections through both blisters from the previously infested guinea pig. Beneath the fibrin is an enormous leucocytic mass (cracked in the process of sectioning) which is already walled off by a new growth of epithelium. Although the one previous infestation with larvae had almost no effect on the percentage of nymphs engorged or their weight, it did greatly accelerate the foreign body reaction around the site of attachment.

Probably, in animals repeatedly infested with nymphs, this reaction is still more rapid and extensive, accounting for the abnormally small size of nymphs engorged on such animals.

DISCUSSION

I. An effective acquired immunity to larvae of the tick, *Dermacentor variabilis*, can be induced in guinea pigs by a single infestation with larvae or nymphs. This immunity develops to its fullest extent within about two weeks after the beginning of the first infestation and lasts at least three months. The immunity is a general one. A local immunity, however, also plays some part, as during the course of establishment of the immunity, regions which have been infested by ticks show a greater resistance than previously uninfested regions. The immunity can be passively transferred by the inoculation of serum, showing that circulating antibodies are concerned in the immune mechanism. On the basis of the cellular reactions in non-immune and immune guinea pigs, it seems reasonable to conclude that the circulating antibodies greatly accelerate and intensify the foreign body reaction at the site of attachment of the larval tick and so wall it off from its blood supply before it can become engorged. Repeatedly, I have observed a mass of larvae attach on the ear of an immune guinea pig and begin to engorge. By the third or fourth day, however, a large blister would be present at the region of attachment of the ticks. Most of the ticks would then gradually shrivel and die while still attached. Generally, a few small pale-colored ones would detach themselves and survive. It is possible that there is also some immune mechanism in addition to this accelerated and intensified foreign body reaction. On very solidly immune guinea pigs, much fewer larvae appeared to attach in the first place than on partially immune or susceptible animals. Since observations were not made at intervals of less than one day, perhaps the larvae did attach and then quickly detached. At any rate, the cellular reaction itself can account for the observed completely effective immunity to the larvae and for the reduction in the amount of blood taken by nymphs and adults from immune animals.

The acquired immunity to ticks thus involves essentially the same mechanism which is apparently concerned in all known immunity to metazoan parasites and possibly to all infectious agents—an antibody accelerating a local reaction in strategically placed organs (Taliaferro, 1934). The cellular reactions of immune animals to certain helminth parasites provide several interesting similarities to the reactions at the tick bites. The experiments of Sarles and Taliaferro (1936) and Taliaferro and Sarles (1937) with the nematode *Nippostrongylus muris* show that, whereas in the skin of normal rats migrating larvae incite a mild temporary diffuse inflammation, in the skin (and lungs) of immune rats they are temporarily immobilized and are surrounded by cell accumulations. The immunity is passively transferable but acts locally in certain organs. Turner, Dennis and Berberian (1937) have found that in sheep previously injected with hydatid antigen the cysts of *Echinococcus granulosus* are effectively walled off and are much smaller than in untreated animals. Kerr (1938a) has likewise observed the rapid walling off of pig ascaris larvae in immunized guinea pigs and has obtained some evidence (1938b) for the presence of circulating antibodies.

II. The question as to what is the actual antigenic substance injected by the attached ticks is of considerable interest. Since the tick's mouthparts are the only portion of it coming in contact with the host's tissues, it seems very probable that the antigenic substance is injected during the process of feeding and is some material present in the salivary secretion. Nuttall and Strickland (1908), Cornwall and Patton (1914), Khodukin and Sofiev (1931) and Hoeppli and Feng (1933) have all demonstrated the presence of powerful anticoagulins in the salivary glands of ticks. Possibly the anticoagulin functions as antigen. Cornwall and Patton (1914), however, could not induce in rats or rabbits the formation of an antienzyme to the anticoagulin of the fly *Philaematomyia insignis*.

There is some evidence that the salivary secretions of blood-sucking insects act as antigens. Boycott (1926, 1928) and Peacock (1926) report observations indicating that the wheal, resulting from the bite of certain insects on susceptible persons, forms only if the person has been previously bitten by the same kind of insect. The first bite produces a sensitization and the wheal following subsequent bites is in the nature of an allergic reaction. Lester and Lloyd (1928) have shown that tsetse-flies from which the salivary glands have been rem[illegible]d are able to live and take blood meals for some time but produce no skin reaction even in very susceptible persons.

III. It has long been popularly held that the natives of regions heavily infested by blood-sucking arthropods are not bothered by them nearly as much as newcomers. Nuttall and Strickland (1908) state that strangers to a district suffer more severely than natives from tick and mosquito bites. The question still remains: are the resistant natives not troubled by the arthropod because it does not bite them, or because, although they are bitten as frequently as non-resistant strangers, they do not react to the bites? Either or both phenomena may well occur with different species of arthropods. Rozeboom (1936) remarks that the donor of blood to a laboratory colony of *Anopheles albimanus* soon became immune to the effects of the mosquito bites, although the insects presumably continued to feed on him as well as ever.

According to Ross (1926), there is evidence that dogs living for many years in tick-infested places in Australia rarely suffer from tick paralysis. Tzortzakis and Papadakis (1936) similarly note that in Crete young sheep and goats are much more susceptible to tick paralysis than older ones. The field observations of Johnston and Bancroft (1918) on a tick-resistant condition of certain cattle in Queensland, Australia, furnish the only previously reported evidence which indicates the possibility of an acquired resistance to ticks themselves. Johnston and Bancroft repeatedly observed that cows of different breeds which were heavily infested with *Boophilus australis* Fuller during one year in the paddock, or were allowed to run wild (and hence probably heavily infested also), were subsequently resistant. Larval ticks would attach, but few were able to mature. The resistant cows frequently showed a yellow exudate on various parts of the skin during the tick season and formed, at the site of attachment of a tick, a peculiar scab never seen on susceptible animals. The tick attached at the region of such a scab dried up and was shed with the scab. The similarity between this reaction and that in guinea pigs immune to *D. variabilis* suggests that the resistance of cattle to *B. australis* observed by Johnston and Bancroft was an acquired immunity of the same type as that exhibited by guinea pigs to *D. variabilis*. On the other hand, Legg (1930) did not observe any increased resistance of cattle to the cattle tick *Boophilus australis* following prolonged exposure.

Most of the work on immunity to metazoan parasites, beginning with that of Sandground (1928) and Stoll (1929), has been concerned with helminth parasites (see Culbertson, 1938, for a review of the literature). But as long ago as 1919 Reuling showed that fish could acquire immunity to the glochidia of fresh water mussels. Blacklock and Gordon (1927) and Blacklock, Gordon and Fine (1930), in one of the earliest detailed studies on immunity to metazoan parasites, found that guinea pigs acquired immunity to larvae of the fly *Cordylobia anthropophaga*. These larvae burrow in the subcutaneous tissues of their host. The only ectoparasites, other than ticks, to which the development of an acquired immunity has been demonstrated are the glochidia already mentioned and *Epibdella mellani*, a trematode parasite of marine fish (Nigrelli and Breder, 1934). The present paper furnishes the first experimental evidence for the development of a true acquired immunity to a blood-sucking arthropod. It thus extends to yet another group of parasites the classical concepts of immunology which, during the past ten years, have been shown to apply as much to metazoan as to bacterial and protozoan parasites.

SUMMARY

One infestation of guinea pigs or rabbits with larvae of the American dog tick, *Dermacentor variabilis*, induces an acquired immunity which effectively prevents subsequent batches of larvae from engorging. In guinea pigs, the immunity develops fully within two weeks after the start of the first infestation and lasts at least three months. Guinea pigs first infested with either *D. variabilis* or *D. andersoni* show a cross immunity to larvae of the other species. Similarly, rabbits first infested with either *D. variabilis* or *Haemaphysalis leporis palustris* show cross immunity to larvae of the other species. Deer mice become relatively resistant to larvae of *D. variabilis* after two or three infestations.

The repeated infestation of guinea pigs with nymphs or adults of *D. variabilis* results in a marked reduction in the amount of blood taken by ticks of the later batches.

The immunity of guinea pigs to larvae of *D. variabilis* can be pro-

duced artificially by the intracutaneous inoculation of an extract of larval ticks. It can be passively transferred by the intraperitoneal inoculation of serum from guinea pigs hyperimmunized by repeated infestations with nymphs.

In the ears of non-immune guinea pigs, on the fourth day after attachment of a larva of *D. variabilis*, there is little cellular reaction at the site of attachment. In immune animals, there is present by the fourth day an intense inflammatory reaction. The mouthparts of the tick are met by a solid mass of leucocytes and the epithelium has thickened and begun to grow beneath the leucocytic mass. In this way, the tick becomes walled off from its source of supply of blood before it can engorge.

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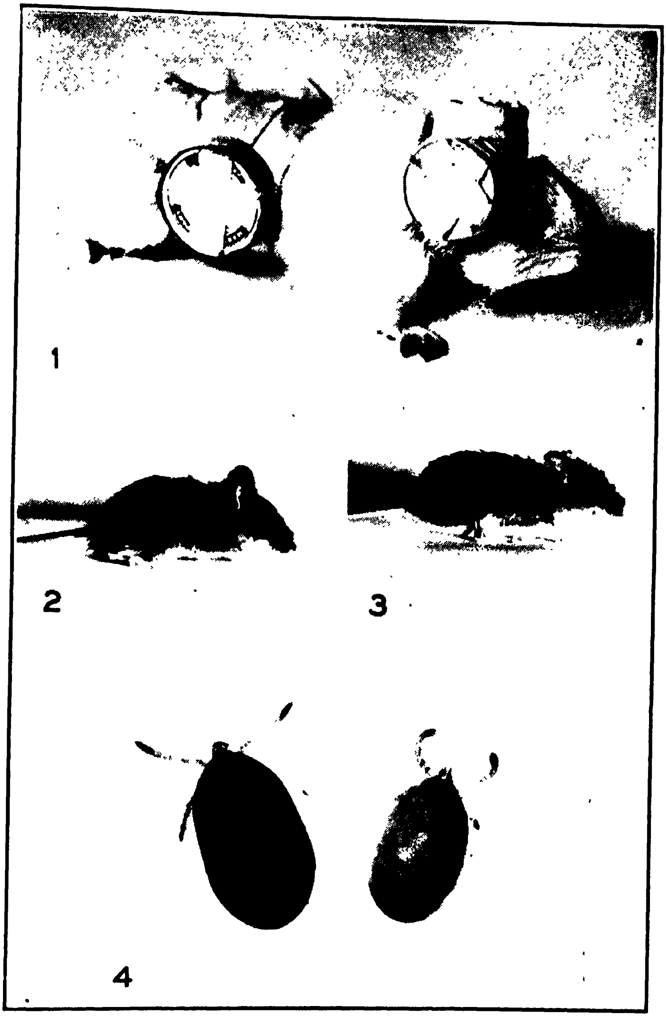

Plate I

Photographs by J. Carlile.

Fig. 1. Guinea pig with pill boxes attached on sides and over ears.

Fig. 2. Deer mouse 2 on the fifth day of its fourth infestation with larvae of *D. variabilis*.

Fig. 3. Deer mouse 6 on the fifth day of its first infestation with larvae of *D. variabilis*.

Fig. 4. Normal engorged larva of *D. variabilis* (left) and a very pale brown engorged larva (right). ×17.

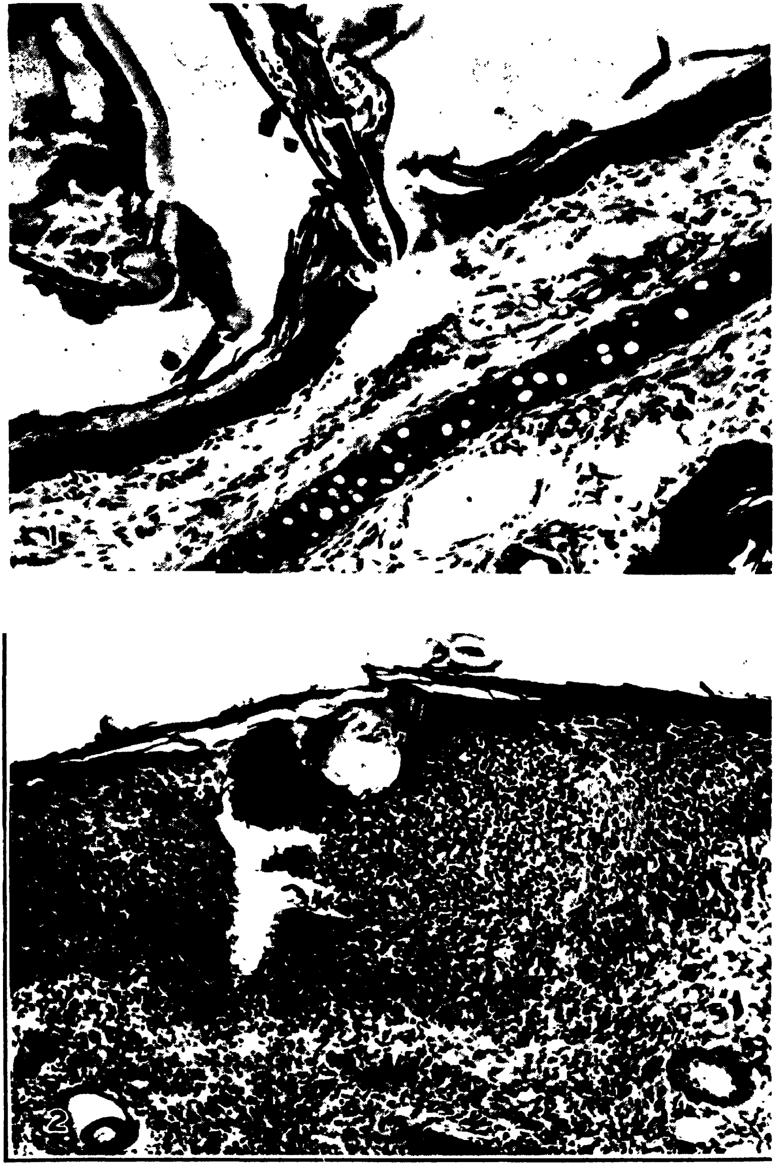

Plate II

Sections through the point of attachment of a larva of *D. variabilis* on the ear of a susceptible guinea pig (Fig. 1), and an immune guinea pig (Fig. 2). Both ×150. Giemsa stain. (Photographs by J. Carlile.)

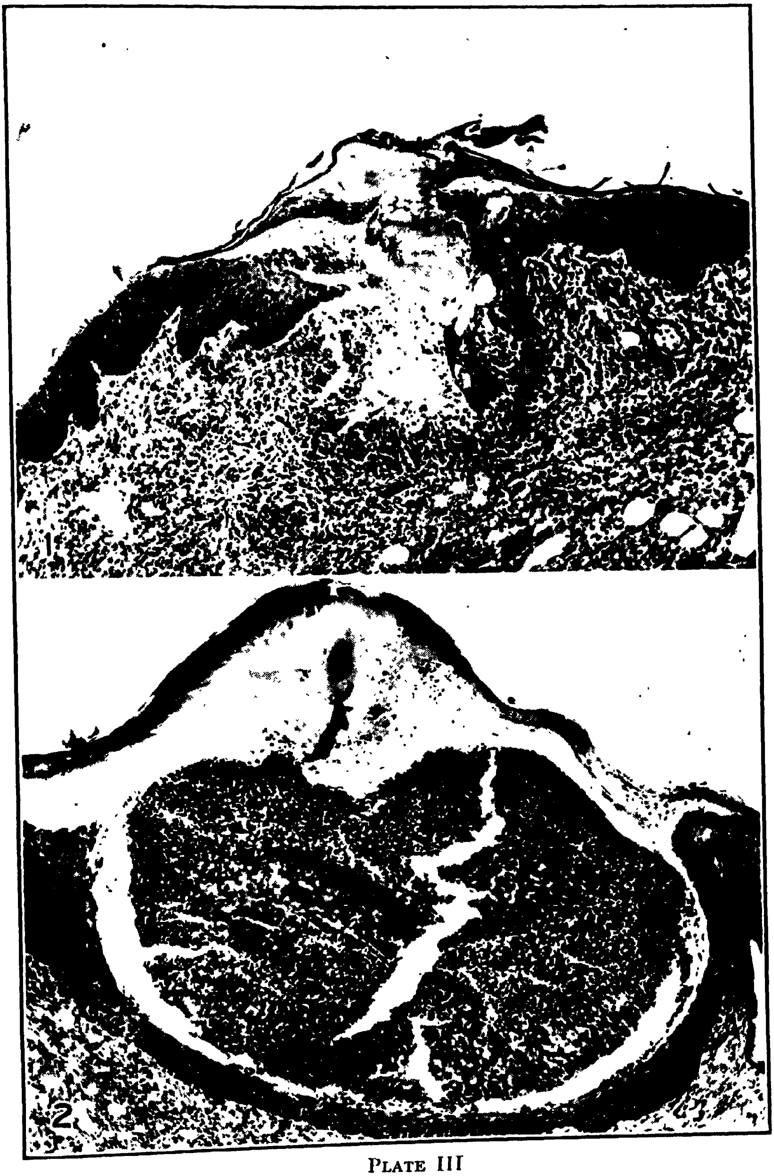

PLATE III

Section through a nymphal tick bite on the ear of a previously uninfested guinea pig (Fig. 1) and a guinea pig previously infested with larvae (Fig. 2). Both ×97. Giemsa. (Photographs by J. Carlile.)

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FURTHER OBSERVATIONS ON ACQUIRED IMMUNITY TO THE TICK DERMACENTOR VARIABILIS SAY

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(Received for publication, October 12, 1938)

It has been shown (Trager, 1939) that guinea pigs and rabbits once infested with larvae of the tick *Dermacentor variabilis* acquire an effective immunity against these larvae. This immunity can be produced artificially by the inoculation of an extract of larval ticks and can be passively transferred. It depends to a large extent on the presence of a circulating antibody which accelerates a local cellular reaction which in turn walls off the tick larva from its normal food supply. Some additional information has now been obtained concerning two of the points left open in the previous paper.

I. *Immunization with various tick extracts.* Guinea pigs were inoculated intracutaneously on the left side with various tick tissue extracts, or with 0.85 per cent salt solution alone. By means of the pill box method previously described, larval ticks from a single batch of eggs were then applied to both sides and to the left ear of all the guinea pigs. Table 1 shows the results of such an experiment. It is clear that antigenic substance is present in the digestive tract and the cephalic glands of partly engorged ticks, as well as in the salivary glands of both fed and unfed ticks and in whole larval ticks. The cephalic gland extract, however, is not nearly as potent as the salivary gland extracts. It is worthy of note that during the course of the inoculations guinea pigs 4 and 6 showed marked reddening and hardening of the skin at the site of injection; guinea pigs 5, 9 and 10 showed a similar but milder reaction; while the other animals showed no local reaction whatever. Nevertheless, guinea pig 2 showed more immunity than guinea pig 4.

II. *The complement fixation reaction.* In the previous paper it was noted that guinea pig serum could not be used to demonstrate specific

TABLE 1

Immunization of Guinea Pigs with Various Tick Extracts

	Guinea pig	Injected intracutaneously on left side:			Number of larvae engorged out of 100 applied on Sept. 22, 1938, to each of following regions:			Total number of engorged larvae
		Amount	On Sept. 10, 11, 12 with	On Sept. 13, 14 with				
			Extract*	Extract*	Left side	Right side	Left ear	
		cc						
Litter mates	1—♀	0.1	CG—1	CG—2	24	22	29	75
	2—♀	0.1	SG—1	SG—2	9	9	5	23
	3—♂	0.1	S	S	41	54	71	166
	4—♂	0.05	L	L	1	33	29	63
Litter mates	5—♀	0.1	SG—3	SG—4	3	13	9	25
	6—♀	0.1	L	L	0	13	11	24
	7—♀	0.1	S	S	46	47	22	115
	8—♂	0.1	SG—1	SG—2	36	25	36	97
Litter mates	9—♂	0.1	DT	DT	29	29	..	58
	10—♂	0.1	SG—3	SG—4	30	32	..	62
	11—♂	0.1	S	S	25	66	..	91

* The extracts were prepared by grinding various tissues in salt solution, as shown below. All the suspensions were centrifuged and the slightly turbid supernatant liquids used.

Extract	Tissue	Stage of tick	No. of ticks used	Vol. of 0.85% NaCl solution	Date of preparation
				cc	*September*
CG—1	Cephalic glands	Half-engorged adult ♀	4	1	9
CG—2	" "	" "	6	1	12
SG—1	Salivary glands	Unfed adult ♀	4	2	9
SG—2	" "	" "	12	1	12
L	Entire body	Larva	2000 approx.	6	9
SG—3	Salivary glands	Half-engorged adult ♀	4	2	9
SG—4	" "	" "	5	1	12
DT	Digestive tract	" "	4	2	9
S	0.85 per cent saline alone				

complement fixation because normal guinea pig serum reacted with tick extract antigen to fix complement. This difficulty has been overcome by using rabbit serum. Table 2 shows a typical result.

The complement fixation technic recommended by Taliaferro (1929) was followed. The reagents used were:

Antigen: Approximately 0.2 gm. of larval ticks ground up in 2 cc. of 0.85 per cent saline solution. Mixture centrifuged and supernatant diluted with saline to 25 cc.

TABLE 2

Complement Fixation in the Presence of Tick Extract Antigen and Serum from Rabbits Previously Infested with Ticks

Rabbit No.	Previous treatment	Serum dilution	Degree of complement fixation
1—♂	77 larvae engorged on rabbit June 7–14, 1938. Bled on Sept. 6, 1938.	1:4	+++
		1:8	++
		1:16	+
		1:32	±
		1:64	−
2—♂	44 nymphs engorged on rabbit Sept. 6–15, 1938. Bled on Sept. 22, 1938.	1:4	++++
		1:8	+++
		1:16	++
		1:32	±
		1:64	−
3—♂ Litter mate of 2	84 nymphs engorged on rabbit Sept. 6–15, 1938. Bled on Sept. 22, 1938.	1:4	++++
		1:8	++++
		1:16	+++
		1:32	++
		1:64	−
4—♂ Litter mate of 2	No ticks. Bled on Sept. 22, 1938.	1:4	±
		1:8	±
		1:16	±
		1:32	−
		1:64	−

Amboceptor: Rabbit serum hemolytic for sheep cells in presence of complement.

Complement: Mixed serum from 3 normal guinea pigs.

Sheep red cells: These were washed 3 times with saline.

Test serums: Obtained from rabbits and inactivated by heating for one-half hour at 55° C.

The amboceptor was titrated first. On the basis of this titration a mixture of sheep cells, amboceptor and saline (so-called sensitized cells) was prepared such that the addition of 0.1 cc. of it to 1.9 cc. of other constituents would introduce two units of amboceptor and would give a red cell concentration of 0.5 per cent. In the presence of these concentrations of amboceptor and cells, the complement was then titrated. In the actual complement fixation tests, two units of complement, in this case 0.2 cc. of a 1:10 dilution, 0.7 cc. of antigen and 1 cc. of the appropriate dilution of test serum were mixed and incubated one hour at 37°C. (In such a mixture the guinea pig serum used for complement was present at a dilution of 1:100, whereas 1:32 was the highest dilution of normal guinea pig serum which gave more than a trace of the non-specific complement fixation in the presence of tick extract.) Then 0.1 cc. of sensitized cells was added to each tube, and the tubes were again incubated one hour at 37°C. Suitable controls showed that neither the antigen alone nor the test serums, at the concentrations used, had any anticomplementary action.

As shown in table 2, normal rabbit serum combined with tick extract did give a slight complement fixation (± indicates a trace of unhemolyzed red cells present), but this was negligible as compared with the high degree of complement fixation exhibited by the serums from previously infested rabbits, two of which were litter mates of the uninfested control.

SUMMARY

Guinea pigs can be partially immunized to larvae of *Dermacentor variabilis* by injecting them with extracts of the cephalic glands, salivary glands, or digestive tract of partially engorged adult female ticks, or of the salivary glands of unfed adult females.

The serum of rabbits previously infested with *D. variabilis* shows specific complement fixation with a larval tick extract antigen.

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THE ANTIBODY RESPONSE TO SWINE INFLUENZA*

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(Received for publication, November 4, 1938)

It is well known that antibodies capable of neutralizing swine influenza virus are present in the sera of swine recovered from swine influenza (1–4). However, neither the exact time following infection that these antibodies first appear nor the time at which they reach their highest titer has been determined. The present experiments were conducted in order to obtain this information.

Methods

Five swine were inoculated intranasally with a mixture of strain 15 swine influenza virus (1) and *Hemophilus influenzae suis* (5). Four of these animals developed typical swine influenza, while the 5th had an extremely mild illness like the "filtrate disease" seen in swine infected with virus alone (1). A 6th pig was inoculated intranasally with swine influenza virus alone and developed filtrate disease. These 6 swine were bled just prior to infection and then repeatedly during illness and after recovery, and the sera thus obtained were titrated for antibodies which neutralize swine influenza virus.

Titration of Antibodies for Swine Influenza Virus.—The neutralization tests were conducted in the usual way in white mice (3), employing the supernatant of a 2 per cent suspension of glycerinated infected mouse lung as virus and mixing this in equal parts with the serum dilution to be tested. Strain 15 swine influenza virus was used in all tests. The serum dilutions were prepared in physiologic salt solution, using 0.2 cc. of serum in varying amounts of the diluent. A further twofold dilution occurred, when the serum was added to the virus suspension. Three etherized mice were inoculated, in testing each serum dilution, by dipping their noses in the virus-serum mixture contained in a slightly tilted small Petri dish. Each neutralization experiment was allowed to run for 10 days. Mice which succumbed during the 10 day observation period and showed typical pulmonary pathology of influenza at autopsy were considered to have received a non-neutralizing dilution of serum. Those which survived the 10 day period were considered to have received a neutralizing dilution of serum. The final titer of a given serum was taken as the highest dilution which protected all or the majority of the mice against death.

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*This paper is part of a thesis submitted by Dr. Carlos T. Rosenbusch to the Faculty of Iowa State College in partial fulfillment of the degree of Doctor of Philosophy.

RESULTS

Swine 1993 (Fig. 1) developed typical swine influenza of 7 days' duration, and neutralizing antibodies were first detectible in its serum on the 6th day after infection. They rose to a titer of 1:20 on the 7th day and remained at this level until the animal was killed on the 11th day.

Swine 1974 (Fig. 2) was ill of swine influenza for 7 days following inoculation, and neutralizing antibodies first appeared in the serum on the last day of illness. By the 10th day the antibody titer had reached 1:20, and a titer of 1:60 was attained on the 14th day. Two days later it had decreased to 1:40, and this level was maintained until the termination of the experiment on the 84th day.

Swine 1975 (Fig. 3), inoculated in the same manner with a mixture of swine influenza virus and *H. influenzae suis*, did not show the usual clinical manifestations of swine influenza. Instead it underwent only a brief mild illness characterized clinically by malaise and inappetence of 2 days' duration. The clinical picture was indistinguishable from filtrate disease seen in swine infected with swine influenza virus alone. Infections of this type are of extremely rare occurrence in fully susceptible swine. The exact time at which neutralizing antibodies first appeared in the serum of this pig is unknown, because bleedings were unfortunately omitted on the 8th and 9th days after inoculation. No antibody was detectible on the 7th day, while by the 10th day the titer had reached 1:40. By the 14th day the titer had risen to 1:60, and this level was maintained through the 21st day. On the 27th day the antibody titer was found to have risen to 1:120, and this level was maintained through the 46th day. It had decreased to 1:80 on the 62nd day and was still 1:80 on the 84th day when the experiment was terminated.

Swine 1984 (Fig. 4) was ill of swine influenza for 6 days, and neutralizing antibody first became detectible in its serum on the 7th day. By the 10th day the titer had risen to 1:20 and on the 14th day it reached 1:40. By the 20th day the antibody titer was 1:120. At this time the animal was tested for active immunity to swine influenza by intranasal inoculation with a mixture of swine influenza virus and *H. influenzae suis*. It proved solidly immune. Serum was obtained 6, 12, 24, 50, and 72 hours following the immunity test and titrated for neutralizing antibody, but no significant change in the antibody titer was observed. The titer was still 1:120 when the experiment was terminated on the 31st day.

Swine 1985 (Fig. 5) had a characteristic swine influenza of 7 days' duration, and neutralizing antibody first appeared in the serum on the last day of illness. The titer reached 1:20 on the following day and by the 10th day had risen to 1:80.

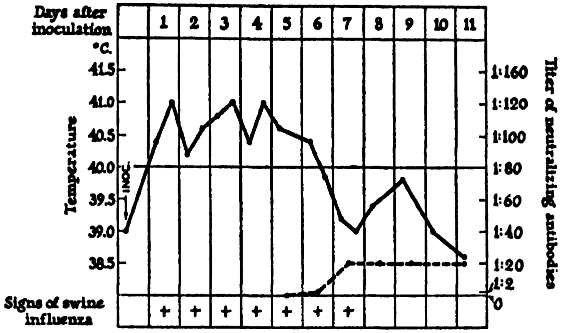

FIG. 1. Swine 1993. Inoculated intranasally with swine influenza virus + *H. influenzae suis.*

Figs. 1 to 6. The full line represents temperature; the broken line, neutralizing antibody titer.

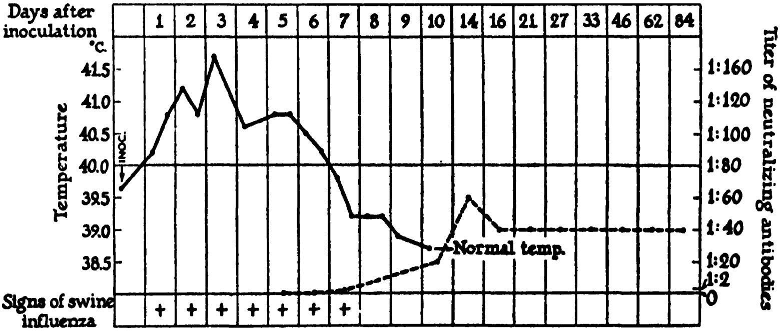

FIG. 2. Swine 1974. Inoculated intranasally with swine influenza virus + *H. influenzae suis.*

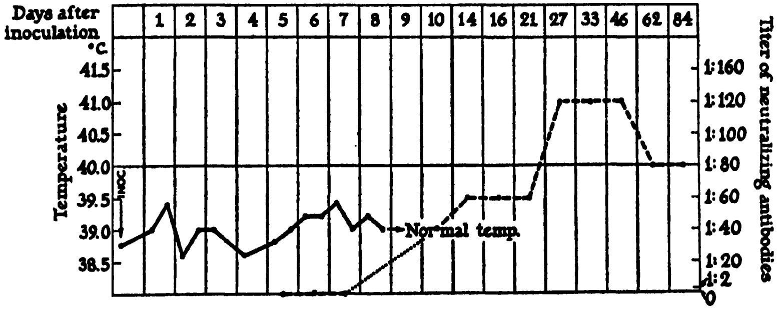

FIG. 3. Swine 1975. Inoculated intranasally with swine influenza virus + *H. influenzae suis.*

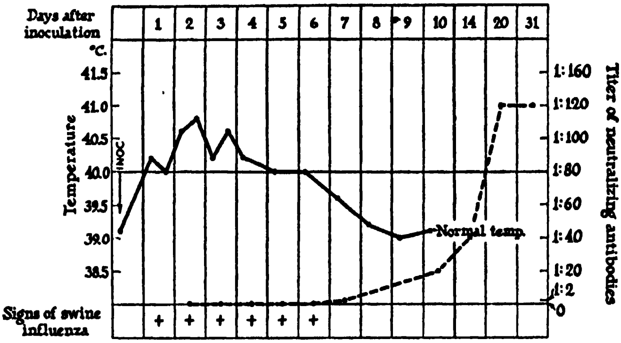

FIG. 4. Swine 1984. Inoculated intranasally with swine influenza virus + *H. influenzae suis.*

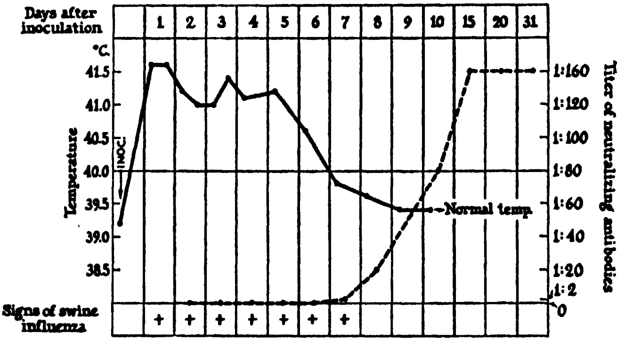

FIG. 5. Swine 1985. Inoculated intranasally with swine influenza virus + *H. influenzae suis.*

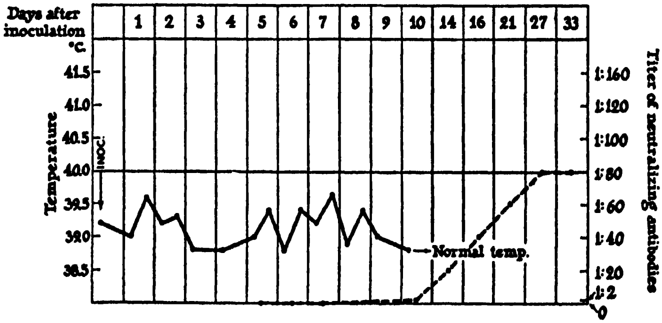

FIG. 6. Swine 2002. Inoculated intranasally with swine influenza virus alone.

On the 15th and 20th days the antibody titer was 1:160. The animal was tested for active immunity to swine influenza on the 20th day and found to be solidly immune. Serum drawn 6, 12, 24, 50, and 72 hours after the immunity test was titrated for antibody, but no significant change was found. The titer remained at 1:160 when the experiment was concluded on the 31st day.

Swine 2002 (Fig. 6) was infected with swine influenza virus alone and underwent an attack of the mild filtrate disease. There was no significant temperature elevation, and clinically the illness was characterized by malaise and inappetence of 2 days' duration. Neutralizing antibody first became detectible in the serum of this animal on the 10th day. The antibody titer rose gradually to 1:80 on the 27th day and persisted at this level on the 33rd day when the experiment was terminated.

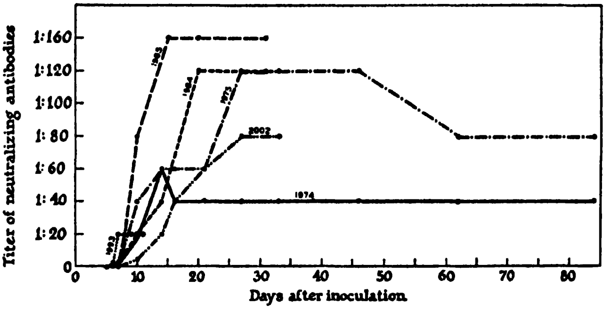

FIG. 7. Comparative antibody response of the 6 swine to swine influenza.

DISCUSSION

The results summarized in Fig. 7 illustrate the variable extent of the antibody response of individual swine to swine influenza. In the 4 animals that underwent typical attacks of the disease, antibodies were present by the 7th day after infection, while in the 2 that suffered only the mild filtrate disease, antibodies did not appear until sometime after the 7th day. Similarly the time required to reach the highest antibody titer appeared to be influenced by the clinical severity of the disease. Excluding swine 1993, observed for only 11 days, the animals with typical influenza reached their maximum titers on the 14th, 15th, and 20th days after infection. The 2 swine that underwent attacks of the mild filtrate disease, on the other hand, did not reach their maximum titers until the 27th day after infection. There was no apparent relationship between clinical severity of disease and the maximum antibody titers eventually reached. These ranged from 1:60 to 1:160. In 2 animals kept under observation for 84 days there was some decrease in titer from the highest level attained.

The present findings concerning the antibody response in swine influenza are similar to those noted by investigators of human influenza. Smith and Stuart-Harris (6) observed that the antibody titer of a human case had risen considerably by the 8th day after onset of illness, reached a peak between the 16th and 31st days, and had declined slightly by the 44th day. Francis and his coworkers (7) noted, in another human case, that the antibody titer rose abruptly on the 7th day, reached a peak on the 14th day, and then gradually declined. Smorodintseff and his coworkers (8) reported 25 to 100-fold increases in the neutralizing antibody titers of the sera of their volunteers 10 to 15 days after experimental infection with human influenza virus.

In the 4 swine that underwent typical attacks of swine influenza, the appearance of neutralizing antibodies coincided rather closely with defervescence and clinical recovery, suggesting that the antibodies may have contributed materially to the cessation of signs of illness. It is known, furthermore, that swine influenza virus is, as a rule, no longer demonstrable in the swine respiratory tract 7 or more days after infection. The anatomical changes produced in the lung by the infection, however, persist for a variable period of time after recovery is clinically apparently complete. The possibility that the appearance of circulating virus-neutralizing antibody is the sole cause of clinical recovery is rendered unlikely by the findings in the cases of the 2 mildly ill animals, in which, though signs of clinical infection persisted for only 2 days, neutralizing antibody did not become detectible until after 7 days.

SUMMARY

Antibodies that neutralize swine influenza virus became detectible in the serum of swine on the 6th or 7th day after infection with swine influenza. Their appearance corresponded rather closely with clinical recovery. In swine with the milder filtrate disease, neutralizing antibodies did not appear until sometime between the 7th and 10th days. The maximum antibody titers ranged from 1:60 to 1:160 and were attained on from the 14th to the 27th days after infection.

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POTENTIALLY UNLIMITED GROWTH OF EXCISED PLANT CALLUS IN AN ARTIFICIAL NUTRIENT

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(Received for publication, December 23, 1938)

One of the ultimate objects of the cultivation of isolated plant or animal organs or tissues has always been, as Haberlandt (1902) long ago pointed out, the investigation of problems concerning the metabolism of the individual cell and its relation to neighboring cells. This object can be fully attained only if the cell can be isolated from its fellows, either anatomically or physiologically. Haberlandt and his immediate pupils sought to isolate cells anatomically. But the same end can be attained approximately if a completely undifferentiated mass of tissue can be grown in which all cells are alike and hence exert like influences on one another. It is for this reason that an "undifferentiated" state has come to be considered one of the irreducible characteristics of a "tissue culture." There is, however, another irreducible characteristic of a true "tissue culture" which must be demonstrated if the metabolism of the cells is to be considered normal. That is unlimited capacity for growth. This question has been repeatedly discussed elsewhere (White, 1931, 1936).

As was pointed out in earlier papers (White, 1934, 1936), the root cultures maintained in this laboratory for the past six years satisfy the second requirement, of unlimited capacity for growth, but are by no means undifferentiated. Gautheret's cultures of excised cambium (1934, 1935, 1938a, 1938b), Nobécourt's carrot root cultures (1937, 1938), and Bonner's pea-pod cultures (1936), on the other hand, are relatively undifferentiated and have the appearance of tissue cultures. Gautheret has grown cambium cultures for more than a year, through five or six passages, thus furnishing evidence of a possible capacity for unlimited growth. It seems probable that he has, therefore, fulfilled both these requirements and is to be credited with the first true plant tissue cultures. His observations, however, do leave some doubt on this score. The tissue fragments used in his cultures were relatively large, one to two centimeters in diameter, and contained considerable stored material. The growth rates reported were so low that transfers could be made only once every 4 to 6 weeks. The cultures may have survived at the expense of the stored nutrients within the explant and may not be capable of unlimited growth. Nobécourt's cultures likewise grew slowly and were of relatively brief duration. Bonner's results were still less satisfactory. A more adequate demonstration is needed than that furnished by the data of these authors.

I have for some time been interested in a hybrid tobacco resulting from the cross *Nicotiana glauca* ♀ × *N. langsdorffii* ♂ (Kostoff, 1930; Levine, 1937). This hybrid has an unusual capacity for proliferation, producing calluses and galls at any point on stem or leaf which has been mechanically abraded. Callus tissue may also develop in the absence of obvious wounding. It seemed possible that tissues of such a plant might be especially favorable for cultivation in vitro. This paper reports the results of an investigation of this possibility.

The nutrient used was the same as that employed for cultures of isolated roots, consisting of a modified Uspenski solution, 2 per cent sucrose and 0.01 per cent of an extract of dried brewer's yeast (White, 1934). It early became evident that most callus tissues would not float on the surface of the nutrient as do roots, so that their oxygen supply might be expected to be inadequate in a liquid nutrient (Zimmerman, 1930; Gautheret, 1935; see also various papers by W. A. Cannon and by D. R. Hoagland and associates). To obviate this difficulty, 0.5 per cent of thoroughly washed agar was added to the above nutrient to make a semi-solid medium. Young stems of the plants were stripped of their leaves. In the laboratory they were broken 4 to 5 cm. back of the tips, where the tissues were brittle enough to break without tearing. Clean, aseptic surfaces were thus exposed. With a sterile scalpel, cones of tissue were removed from the exposed surfaces, the tissue masses consisting of medulla and procambial strands. The excised masses were always less than 5 mm. in greatest diameter. They were placed on nutrient agar in either 125 ml. Erlenmeyer flasks or 40 ml. Pyrex test tubes and set aside in diffuse daylight.

Where such fragments contained only medullary parenchyma, no growth occurred, and the tissues soon turned brown. Wherever procambial strands were present, however, proliferation from these regions began almost immediately. Irregular protuberant masses of

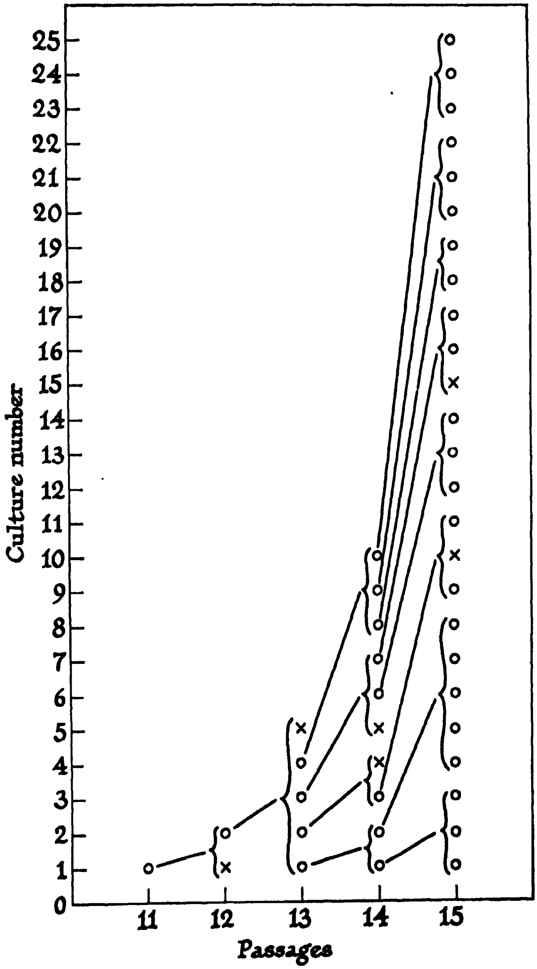

FIG. 1. Diagrammatic representation of the genealogy of the 25 cultures of passage 15, showing their derivation from culture No. 2, of passage 12. Cultures marked with a cross (x) were contaminated with molds or bacteria.

callus were formed, which early took on a bright green color.[1] After from one to two weeks, these masses were cut away from the original explant and transferred to fresh nutrient. The cultures were divided and transferred to fresh nutrient at biweekly intervals through 10 passages. Five rapidly growing cultures were then selected for more detailed study and the remainder discarded. These 5 were divided into 25 fragments, each less than 2 mm. in diameter. Transfers thenceforth were at weekly intervals, and each culture was regularly divided into as many pieces of 1 mm. or less diameter as possible,

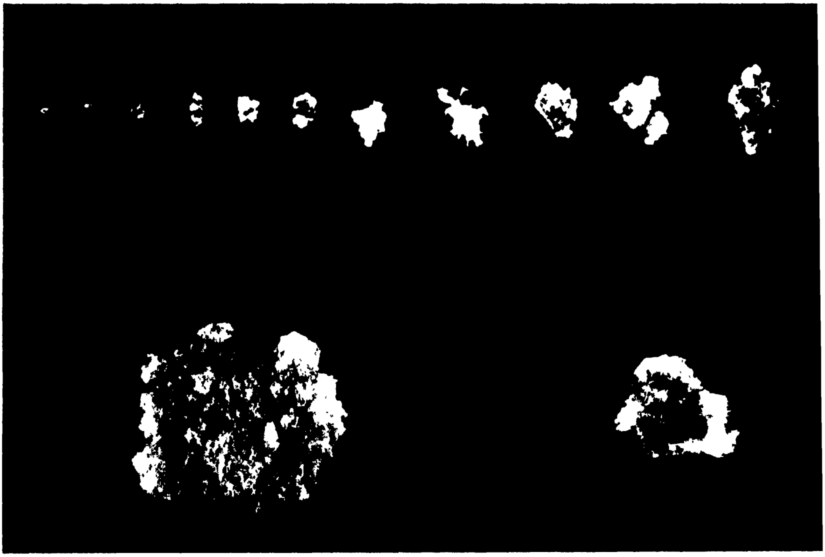

FIG. 2-4.—Fig. 2 (above). A series of cultures of excised hybrid tobacco callus cultivated in vitro. The cultures vary in age from 0 (extreme left) to 10 weeks (extreme right). ×1.7.—Fig. 3 (lower right). Detail of a tissue fragment similar to that shown at the left in figure 2. The surface is irregular, but there is no evidence of differentiation. ×20.—Fig. 4 (lower left). Detail of a 20-weeks-old culture. There is still no macroscopic evidence of differentiation. The tissue mass is so friable as to be easily broken up with a needle. ×2.3. (Photographs by J. A. Carlile.)

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[1] This color was retained in cultures that were subcultured at frequent intervals but disappeared in older cultures, without causing any concomitant reduction in growth rate.

25 cultures being retained in each passage for the next 10 passages. From figure 1 it is clear that by the 15th passage all 25 cultures were derived from culture No. 1 of passage 11 and culture No. 2 of passage 12. The tissue fragment used as No. 2, passage 12, had therefore increased 25-fold in 3 passages. The detailed record shows that each fragment had increased on an average about 3-fold per passage of 7 days, or about 50 per cent per culture per day—an increment rate which compares favorably with that observed in isolated roots. At the end of the 20th passage, the number of cultures was reduced to 10 to conserve space. These cultures had been maintained through 40 passages on November 3, 1938, and are still in excellent condition at the time of writing this paper.

An increase of 3-fold per passage for 40 passages would represent an increase of 3^{40} = ca. 1.2×10^{19}, or a dilution of any material contained in the original explant to a mass of the order of 10^{-19} times its original mass in each culture. It is not to be supposed that any material could be a growth-limiting factor at such a dilution. These cultures, therefore, appear to satisfy the second requisite of a tissue culture, unlimited capacity for growth.

Figure 2 shows a series of cultures prepared by setting aside one culture each week for the ten weeks involved in passages 11 to 20. The oldest piece (extreme right) had thus been undivided for 10 weeks, while the youngest (extreme left) was photographed at the time of subculturing and shows the size of piece used in starting each subculture. Figure 3 shows the detailed appearance of such a culture at the beginning, and figure 4 a similar culture after 20 weeks' growth. There is little if any macroscopic evidence of differentiation. The microscopic structure is in keeping with this appearance. Figures 5 to 13 were all taken from a single transverse section through the center of a culture which had been maintained for 8 weeks without being divided. In general appearance the section shows a central core of tissue resembling medulla (fig. 5, 6) surrounded by an irregular corona of less compact masses (fig. 5, 7). This central core is made up of relatively uniform, moderately large cells with scattered areas of what may have been meristematic tissue and occasional groups of scalariform cells suggesting differentiation into xylem (fig. 6, 8, 9). These xylem elements are isolated and obviously incapable of normal func-

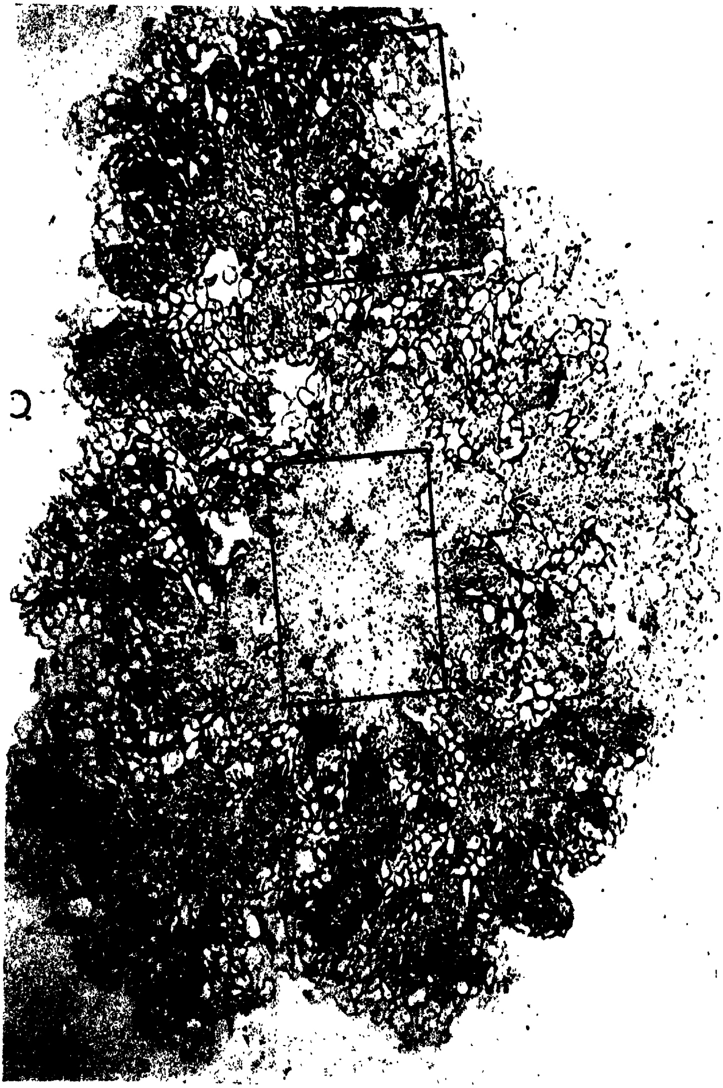


FIG. 5. Transverse section through an 8-weeks-old culture similar to that shown third from the right in figure 2. The central area (see fig. 6) is relatively uniform, while the marginal area (see fig. 7) is less compact and is made up of irregular masses of tissue which might be mistaken for organized growing points or leaves. ×30. (Photograph by J. A. Carlile.)

tion, since they do not extend for any considerable distances through the mass. The younger, peripheral regions show a much wider variation in cell type. The meristematic areas are more frequent (fig. 7, 10, 11) and are obviously functional. Growth thus occurred by means of "growing points" rather than cambium, but these growing regions are not identifiable as "stem" or "root." Growth was random, neither polarized nor resulting in oriented differentiation. The meristematic cells evidently divide for a time, then enlarge (fig. 7, 11, 13), form irregular bands of "giant cells" (fig. 7, 12), and these finally either become thickened (suberized?) (fig. 5, 7) or else are crushed by the new growth. The outermost portions of the tissue mass are usually made up of large, thin-walled cells (fig. 7, 12). The cells throughout the culture contain considerable quantities of starch, although they are nearly if not completely devoid of chlorophyll (fig. 9, 10, 11, 12, 13). Nowhere were the loose "pleurococcoid" growths figured by Gautheret (1934, 1935, 1938b) nor the arcuate cells of Nobécourt observed (Nobécourt, 1937—compare with the sloughed cortical cells of wheat-root cultures, White, 1932, and those observed by Scheitterer, 1931).

These cultures, then, show a behavior similar to that of attached callus but do not undergo anything like the same degree of differentiation (Levine, 1937). It is not possible to identify phloem, cambium, phelloderm, sclerenchyma, nor any other normal cell type except parenchyma, meristem, and an occasional isolated scalariform cell. The cultures, while not quite unorganized, are at least quite disorganized. The tissues are not pith, wood, bark, nor even typical callus. These are not organ cultures. While not strictly comparable to the "pure line" cultures of the animal tissue culturists, they do represent a very close approach thereto. Although all the cells are not quite alike, these masses represent cultures of cells characterized by a very simple and primitive type of growth. It is believed that we have here at last a very near approach to a true plant tissue culture, which should provide us with a new means of approach to the problems of the physiology of the spermatophyte cell.[2]

[2] Since preparing this paper for publication, similar cultures have been successfully established from *Nicotiana tabacum* L., *Lycopersicon esculentum* Mill., and *Beta vulgaris* L., and are now (January 11, 1939) in the sixth passage. This capacity is, therefore, not a special characteristic of the *N. glauca* × *N. langsdorffii* hybrid, but a more general characteristic of normal plants.

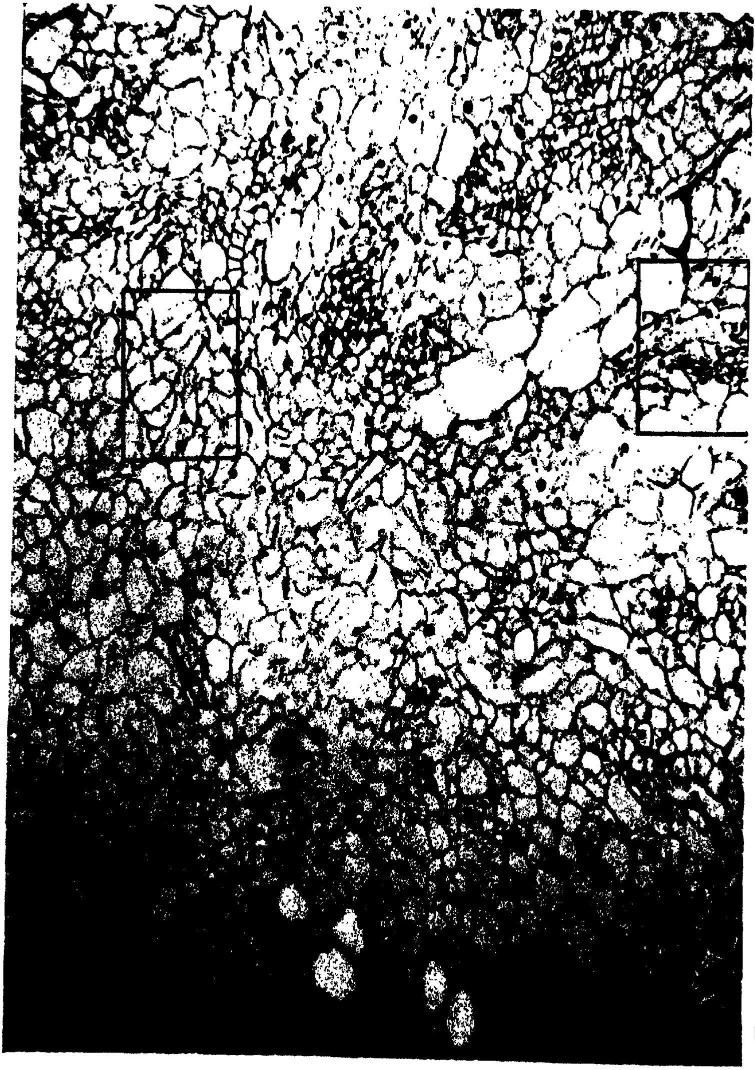


FIG. 6. Detail of the central part of figure 5. The cells of this region are of relatively uniform size, thin-walled, parenchymatous in nature except for scattered denser groups of semi-meristematic cells and occasional scalariform cells (see fig. 8 and 9). ×138.

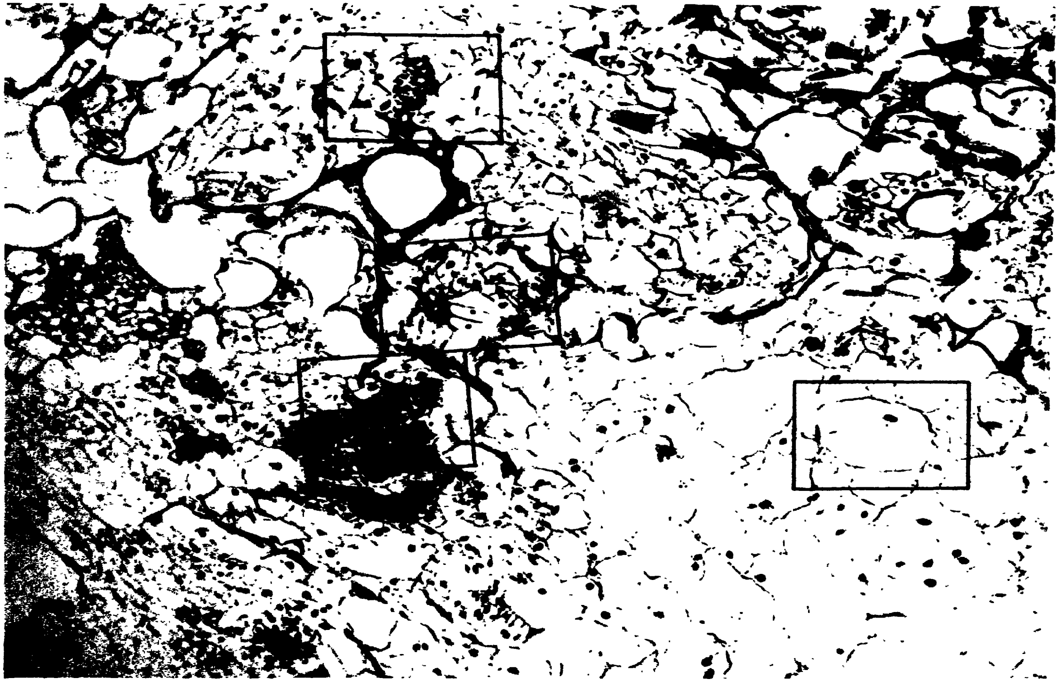
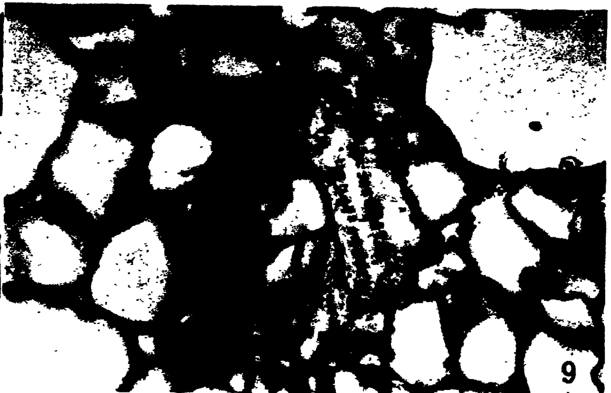


FIG. 7. Detail of a marginal area from figure 5. There are scattered clumps of meristematic cells (see fig. 10, 11) with bands of necrotic tissue, areas of parenchymatous "giant cells," and areas of intermediate type. What at low magnification (fig. 5) appeared to be organized stem growing points appear at higher magnification to be quite unorganized. ×138. (Photographs by J. A. Carlile.)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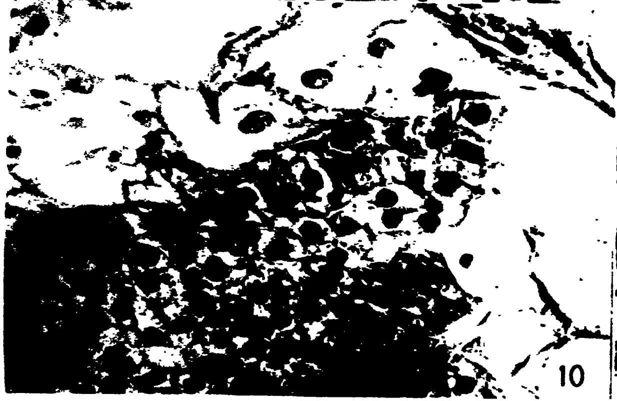
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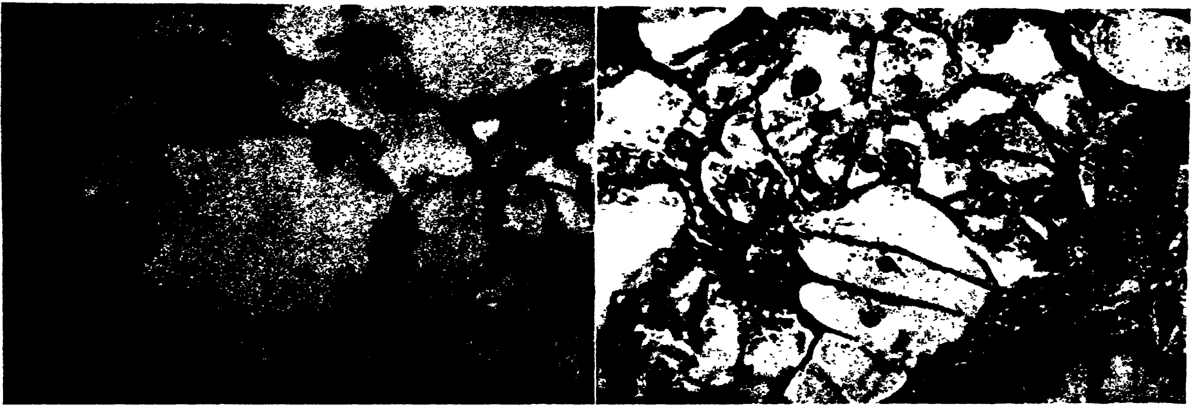

FIG. 8-13.—Fig. 8. Detail of a band of scalariform cells from the upper part of figure 6. ×435.—Fig. 9. Detail of scalariform cells from the lower part of figure 6. ×435.—Fig. 10. Large meristematic area from the center of figure 7. ×435.—Fig. 11. Small meristematic area from the upper part of figure 7. Compare the size of these cells with those in figures 12 and 13. ×435.—Fig. 12. Detail of a "giant cell" from the right hand part of figure 7. Compare in size with the meristematic cells of figures 10 and 11. ×435.—Fig. 13. Detail of a group of cells in figure 7, center, which are obviously derived by division from a small group, possibly 3, of meristematic cells. ×435. (Photographs by J. A. Carlile.)

SUMMARY

Excised callus obtained from proliferating procambial tissue of a hybrid *Nicotiana* (*N. glauca* × *N. langsdorffii*) has been maintained in culture in an environmental complex and nutrient similar to those earlier developed for cultivation of excised roots, through 40 passages of one week duration each. Cultures regularly increased about 3-fold in volume each week, giving a total theoretical increment of 3^{40} = ca. 10^{19}. The conditions can, therefore, be considered adequate for unlimited growth of this material. These cultures show no evidence of differentiation or polarity except for an occasional scalariform cell. Being undifferentiated yet capable of unlimited growth, they appear to satisfy the two main requirements for a true "tissue culture."

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INHIBITION OF VIRUS ACTIVITY BY INSECT JUICES

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INTRODUCTION

In the case of insect vectors in which viruses exhibit an incubation period and in which they are retained for a considerable time, investigations have been made to ascertain if the viruses multiply in the insects. Merrill and TenBroeck (21) have demonstrated that the virus of western equine encephalomyelitis multiplies in the mosquito, *Aedes aegypti* L. Fukushi (14), working with dwarf disease of rice, and Kunkel (17), with aster yellows, interpret their experiments as indicating that the viruses of these diseases multiply in their vectors. Freitag (12) and Bennett and Wallace (3), however, conclude from experiments on curly-top virus that multiplication in the beet leaf hopper is improbable. The potato yellow-dwarf virus has an incubation period in the clover leaf hopper, *Aceratagallia sanguinolenta* Prov. (5), and it is also retained a long time by this vector (6). It was thought that the possible multiplication of this virus in the clover leaf hopper could be investigated by means of the primary lesions that the virus produces in the leaves of *Nicotiana rustica* L. (7). Attempts to obtain primary lesions in *N. rustica* by inoculation with the juice of viruliferous leaf hoppers were, however, entirely negative.

With one exception (20), other investigators (27, 25, 13) have failed in similar attempts to infect plants with juice from insects carrying other viruses. In some cases the failure to obtain infection may be attributed to the fact that the viruses are not readily transmissible from plant to plant by mechanical methods. There has been, however, no satisfactory explanation for the case of other viruses that are transmitted easily from plant to plant but not from insect to plant. Hamilton (15) observed that the addition of juice from crushed aphids, *Myzus persicae* Sulz., to Hy. III virus rendered the mixture noninfectious. She suggested that the insect juice had a deleterious effect on the viability of the virus.

The experiments presented in this paper show that clover leafhopper juice inhibited the infectivity of juice from yellow-dwarf *Nicotiana rustica* plants. The inhibitor (or inhibitors) in the insect juice also suppressed the infectivity of several other viruses, including that of tobacco mosaic. Since yellow-dwarf virus is rather unstable, tobacco-mosaic virus was employed in an investigation of the action and nature of the inhibitor. It was hoped that this study would make it possible to eliminate the inhibitor from the juices of viruliferous leaf hoppers and thus facilitate research on the virus in the insect. Although methods of separating tobacco-mosaic virus from inhibitor were found, the instability of the yellow-dwarf virus has thus far prevented their use with this virus.

Materials and Methods

Clover leaf hoppers, free from extraneous material, were counted, weighed, and inactivated by placing them at –14°C. for 30 minutes. They were then ground in a mortar and the pulp immediately suspended in 0.1 M phosphate buffer at pH 7.0. The suspension was centrifuged to remove the coarse material and the creamy-white supernatant used at once in various experiments. The concentration of the inhibitor in such solutions has been expressed in terms of leaf-hopper weight per cc. For example, the description of a solution as having an inhibitor concentration of 18 mg. per cc. means that 1 cc. of buffer solution contained the juice obtained from 18 mg. of clover leaf hoppers. The average weight of a single leaf hopper varied in different samples from 1.5 to 1.8 mg. Unless otherwise specified, 0.1 M phosphate buffer at pH 7.0 was used in making all suspensions and dilutions. All hydrogen-ion determinations were made by means of a glass electrode.

The infectious juice used in the experiments was prepared from leaves of Turkish tobacco, *Nicotiana tabacum* L., diseased with tobacco mosaic. The leaves were frozen and ground, and the juice expressed through cheese-cloth. The coarser material was removed by centrifugation and the juice stored frozen in small corked test tubes. This juice, or certain dilutions of it in buffer, was mixed with the insect preparation. The effect of different treatments on the inhibitor was determined by the infectivity of the various mixtures tested on from 28 to 36 half-leaves of Early Golden Cluster beans, *Phaseolus vulgaris* L. The samples in each experiment were paired in all possible ways and applied to the half-leaves according to the method of randomization described by Youden (33) as incomplete blocks. In this case, however, the two halves of the same leaf constituted a block. The excess inoculum was rinsed from the leaves with tap water. The lesions were counted 3 to 6 days after inoculation.

EXPERIMENTS

The Nonspecific Nature of the Reaction

As stated in the introduction, the juice from leaf hoppers carrying the potato yellow-dwarf virus was noninfectious to *Nicotiana rustica* leaves. Juice from about 2000 insects was applied to leaves in various ways in the tests. In one experiment each live leaf hopper was placed on a leaf dusted with carborundum, crushed beneath a spatula, and the juices immediately rubbed into the leaf. In control inoculations juice from diseased *N. rustica* leaves produced numerous primary lesions.

In order to determine whether leaf-hopper juice would inhibit the virus, 10 macerated insects were added to each cc. of a 10^{-2} dilution of infectious plant juice. The mixture failed to produce a single lesion on 20 half-leaves of *Nicotiana rustica*, while the corresponding insect-free preparation was highly infectious (Table 1).

It then became of interest to ascertain whether the juice of clover leaf hoppers would inhibit other plant viruses. Suitable dilutions of juices from plants infected with the viruses of tobacco mosaic, potato X, turnip mosaic, tobacco necrosis, or tobacco ring spot No. 1, with and without the addition of 10 macerated leaf hoppers per cc., were compared on opposite half-leaves or opposite leaves of appropriate test plants. Turkish tobacco, *Nicotiana tabacum*; cowpea, *Vigna sinensis* Endl. var. Black; bean, *Phaseolus vulgaris* var. Early Golden Cluster; *N. rustica*; and *N. glutinosa* L. were used in the tests. The results, presented in table 1, indicate that clover leaf-hopper juice has a general inhibitory action. The data demonstrated that the inhibitor is not specific and suggested the possibility of investigating its nature and action by studying its effect on inhibition of tobacco-mosaic virus on bean. Accordingly, tobacco-mosaic virus was employed in all subsequent experiments.

TABLE 1

Inhibiting Action of Clover Leaf-Hopper Juice on Plant Viruses

Virus	Concentration of juice from diseased plants	Test plant	Total lesions in 20 half-leaves or 20 leaves inoculated by virus solution containing:	
			10 leaf hoppers per cc.	No leaf hoppers
Potato yellow dwarf	10^{-2}	*Nicotiana rustica*	0	1156
		Nicotiana rustica	0	180
Tobacco mosaic	10^{-3}	*Nicotiana glutinosa*	5	561
		Bean	0	619
Potato X	10^{-1}	Turkish tobacco	8	845
Turnip mosaic	10^{0}	Turkish tobacco	4	786
Tobacco necrosis	10^{-1}	Cowpea	301	3707
Tobacco ring spot #1	10^{0}	Cowpea	12	1366

TABLE 2

Inhibition of Tobacco-Mosaic Virus by Juice of Various Insect Vectors of Viruses

Insect	Total lesions in 20 half-leaves of bean inoculated with tobacco-mosaic-virus solution containing:	
	15.5 mg. of insect per. cc.	No insects
Aceratagallia sanguinolenta	0	619
Aedes aegypti	0	250
Aphis rumicis	1	647
Eutettix tenellus	0	1885
Macrosiphum pisi	0	461
Macrosiphum solanifolii	0	545
Macrosteles divisus	0	318
Myzus persicae	0	641

Experiments were conducted to determine whether the juice of other insect vectors of viruses likewise contains an inhibitor. The insects used were mosquitoes, *Aedes aegypti*; aphids, *Aphis rumicis* L., *Macrosiphum pisi* Kaltenbach, *M. solanifolii* Ashm., and *Myzus persicae*; and leaf hoppers, *Eutettix tenellus* Baker and *Macrosteles divisus* Uhler. A 10^{-3} dilution of the infectious juice from a mosaic-diseased tobacco plant was added to the macerated pulp of the insect under investigation so that the concentration of the insect juice was 15.5 mg. per cc.—the approximate weight of 10 clover leaf hoppers. This suspension was applied to 20 half-leaves of bean, and the virus preparation to which no insect juice had been added was applied to the opposite half-leaves. Although there was some seasonal variation in the susceptibility of the test plants, the data presented in table 2 indicate that the juices of aphids, leaf hoppers, and mosquitoes have the same inhibiting action on the virus. The results suggest that the inhibitory effect may be a general property of insect juices.

Effect of Dilution on the Inhibitor and the Virus

Dilution studies upon the inhibitor and the virus were undertaken in order to measure the activity of the inhibitor and to elucidate its mode of action. After preliminary tests, a stock solution of the inhibitor was prepared with a concentration of 17.94 mg. per cc. Mixtures were then prepared each of which contained a 1:250 dilution of infectious juice and one or another of various dilutions of inhibitor solution. Each mixture and a 1:250 dilution of infectious juice to which no insect juice had been added was applied to the leaves of 15 bean plants. This and the following experiments are the only ones in which solutions were not tested on half-leaves according to the incomplete block arrangement. The total number of lesions obtained with the solution having no inhibitor was 12,410, and the reduction in the number of lesions obtained with each concentration of inhibitor was calculated as a percentage of 12,410 and plotted in figure 1. It is apparent from the curve obtained that about 0.15 mg. of leaf hopper per cc. reduces the lesions by 50 per cent, and, since the inhibitor doubtless constitutes only a fraction of the leaf-hopper weight, it must be a very active substance. The experiment was repeated twice with similar results.

The full effect of any concentration of inhibitor is exerted immediately after it is mixed with the virus solution. Leaves inoculated with the most concentrated inhibitor solution used showed some injury. These facts suggested that the effect of the inhibitor is on the host plant rather than on the virus. Accordingly, an experiment was designed to determine whether the percentage reduction in lesions depended only upon inhibitor concentration. If the percentage reduction in lesions were independent of virus concentration it would seem certain that the inhibitor acted chiefly upon the plant and little

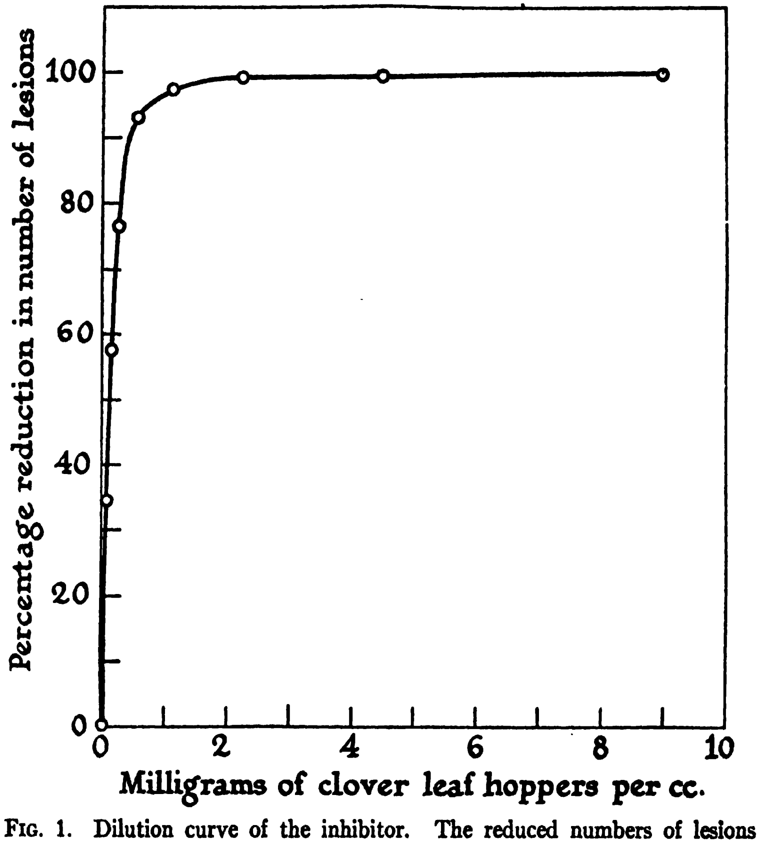

FIG. 1. Dilution curve of the inhibitor. The reduced numbers of lesions produced in 30 bean leaves by a 1:250 dilution of infectious juice in the presence of various concentrations of inhibitor are expressed as percentages of the number (12,410) produced by a 1:250 dilution of the juice without inhibitor.

or not at all upon the virus. A 10^{-1} dilution of the infectious juice was compared by the half-leaf method with the same solution in which the inhibitor concentration was 0.25 mg. per cc.; 10^{-2}, 10^{-3}, and 10^{-4} dilutions, with and without insect juice, were compared in the same way. The data from 3 replications of this experiment are presented in table 3. The general trend of the results is for the percentage reduction to increase slightly as the virus concentration decreases. The virus concentration in the first mixture is 1000 times that of the 4th, yet the percentage reduction varies comparatively little. Although this slight increase in the percentage reduction indicates some action of the inhibitor upon the virus, it seems probable that the principal effect is upon the plant.

TABLE 3

Action of a Single Concentration of Inhibitor (0.25 Mg. per Cc.) on Various Dilutions of Tobacco-Mosaic Virus

Dilution of infectious juice	Experiment 1			Experiment 2			Experiment 3		
	Total lesions in 28 half-leaves of bean		Reduction in lesions	Total lesions in 36 half-leaves of bean		Reduction in lesions	Total lesions in 36 half-leaves of bean		Reduction in lesions
	With inhibitor	Without inhibitor		With inhibitor	Without inhibitor		With inhibitor	Without inhibitor	
			per cent			*per cent*			*per cent*
10^{-1}	2996	6633	54.8	1045	4273	75.5	1226	4645	73.6
10^{-2}	1350	2782	51.5	610	2587	76.4	1160	3050	62.0
10^{-3}	494	789	37.4	177	1075	83.5	366	1146	68.0
10^{-4}	81	272	70.2	41	404	89.9	47	232	79.8

If the principal action of the inhibitor is on the leaf, then dilution of certain virus-inhibitor mixtures should increase their infectivity. A mixture, with a pH of 6.9, containing undiluted infectious juice and an inhibitor concentration of 15.25 mg. per cc. was prepared. Seven dilutions of this mixture in buffer and one dilution of the infectious juice to which no insects had been added were compared on half-leaves of bean. Dilution tests also were conducted with mixtures prepared in the same way but having virus concentrations only 1/10 and 1/100 of that in the first mixture. All 3 mixtures were tested on

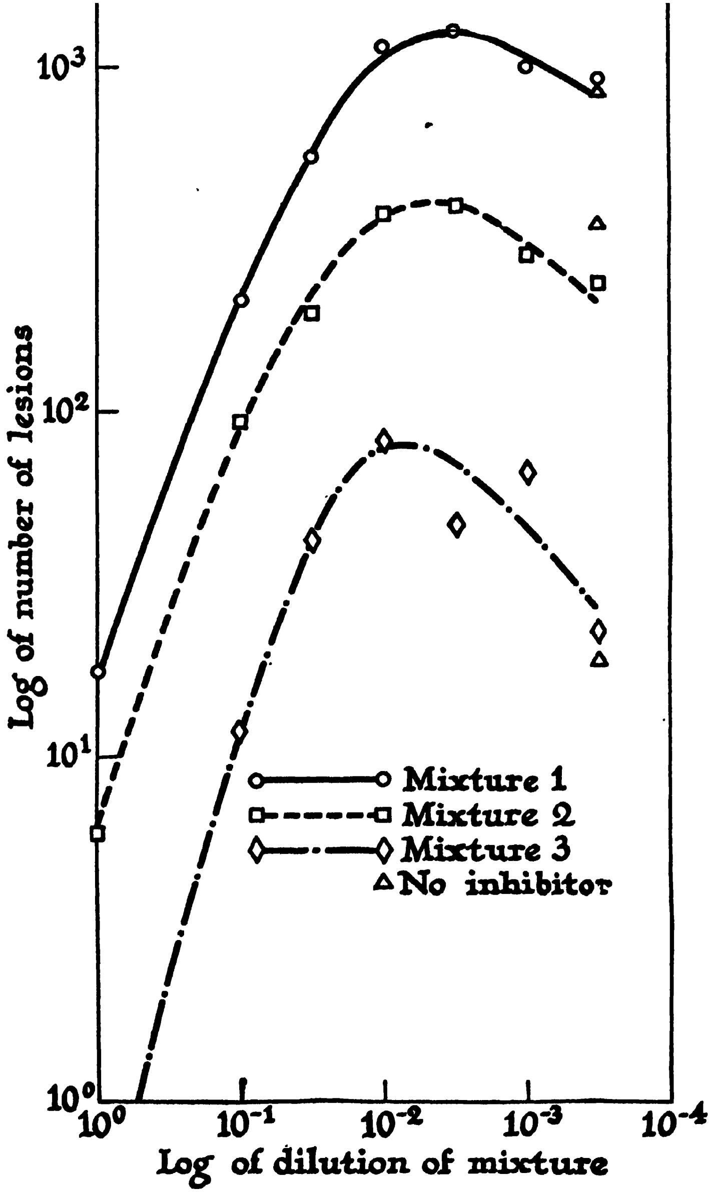

Fig. 2. Increase in the infectivity of virus-inhibitor mixtures upon dilution. All mixtures before dilution contained the same concentration of inhibitor (15.25 mg. per cc.). Mixture 1 contained 100 times as much virus as mixture 3, and mixture 2 ten times as much.

beans from the same lot on the same day, so that the 3 dilution curves obtained (Fig. 2) may be compared one with the other. In each case the infectivity of the mixture increased until the concentration of the inhibitor was only about $10^{-2.5}$ its original concentration, or about 0.05 mg. per cc. At higher dilutions the curve begins to follow the normal dilution curve for the tobacco-mosaic virus. It is interesting that the mixture containing the lowest concentration of virus gave no infections until it had been diluted. The experiment was repeated with similar results, and the same phenomenon was demonstrated, incidentally, in several subsequent experiments. In the light of these results, concentrated suspensions of macerated viruliferous insects would seem less likely to be infective than would more dilute solutions.

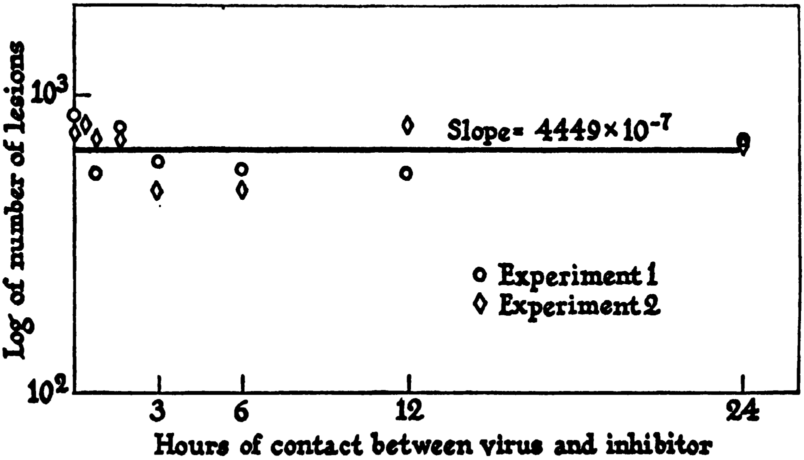

FIG. 3. Stability of tobacco-mosaic virus in the presence of the inhibitor.

The results of the previous experiments indicate that the inhibitor does not destroy the virus during the interval between the preparation of the mixtures and their testing on beans. In order to determine if the inhibitor would destroy the virus during longer intervals of contact, 7 samples of an inhibitor solution with a concentration of 17 mg. per cc. were mixed with equal volumes of infectious juice. After being held for periods up to 24 hours, the mixtures were diluted 1:50 in buffer, and inoculated on bean leaves. The mixtures were prepared at different times but tested for infectivity at the same time. The total number of lesions produced by each sample on 35 half-leaves is plotted in figure 3. The data of a second experiment with 8 samples were adjusted so that the total number of lesions produced by 7 samples was the same as that of the corresponding samples in the first experiment. The corrected values also are plotted in figure 3, and a straight line fitted to all 15 points. The slope of this line (4449×10^{-7}) is not significantly different from zero and indicates there was no appreciable destruction of the virus during 24 hours.

Detection of Virus in Virus-Inhibitor Mixtures

The dilution studies demonstrated that virus could be detected in certain virus-inhibitor mixtures simply by dilution. Other investigations were undertaken for the purpose of finding better methods of detecting virus in noninfectious or slightly infectious virus-inhibitor mixtures. These studies also have contributed information upon the nature of the inhibitor.

Dialysis of the Inhibitor. It seemed possible that the inhibitor could be removed from virus-inhibitor mixtures by dialysis. An inhibitor solution with a concentration of 3.3 mg. per cc. was dialyzed against buffer in a Kunitz-Simms (16) apparatus for 24 hours at room temperature. A control nondialyzed sample of the same solution was stored for 24 hours at room temperature. A third sample consisted of an inhibitor solution of the same concentration freshly prepared when the first sample was removed from the dialyzing apparatus. These solutions were then mixed 1:1 with a 1:125 dilution of infectious juice and the infectivities of the mixtures compared with each other and with a 1:250 dilution of infectious juice to which no inhibitor had been added. If the inhibitor was unaltered by dialysis or storage, the amount used was expected to reduce the infectivity of the virus solution to about 5 per cent of what it otherwise would have been. The total number of lesions obtained on the 36 half-leaves inoculated with each sample is presented in table 4. The data indicate that the inhibitor is not readily dialyzable through a cellophane membrane under the conditions of the experiment and show that the inhibitor is stable over a period of 24 hours. The results of a second experiment, also presented in table 4, were confirmative.

Thermolability of the Inhibitor. An experiment was designed to compare different dilutions of the inhibitor with inhibitor solutions subjected to various temperature treatments. The experiment permitted a quantitative estimation of the effect of the heat treatment on the inhibitor. The temperature treatments were carried out on inhibitor solutions with a concentration of 3.6 mg. per cc. Each solution was added to an equal volume of a 1:125 solution of infectious juice. The 12 solutions in the experiment, therefore, contained a

TABLE 4

Effect of Dialysis upon the Inhibitor

Experiment	Number of half-leaves inoculated	Lesions produced by a mixture containing infectious juice at 1:250 and			
		An inhibitor concentration of 1.65 mg. per cc.			No inhibitor
		Inhibitor freshly extracted	Inhibitor stored at 25° C. for 24 hours	Inhibitor dialyzed 24 hours	
1	36	99	94	231	2277
2	28	127	80	76	1418

TABLE 5

Effect of Heat Treatment upon the Inhibitor

Solution	Concentration of inhibitor (mg. per cc.)	Temperature treatment of inhibitor for 10 minutes (°C.)	Total lesions in 33 half-leaves	
			Exp. 1	Exp. 2
1	1.8	No treatment	55	96
2	0.9	"	338	245
3	0.45	"	557	511
4	0.225	"	1128	1392
5	0.1125	"	1440	1460
6	none	"	1256	2110
7	1.8	100	855	1883
8	1.8	90	917	1395
9	1.8	80	957	1307
10	1.8	70	466	449
11	1.8	60	121	136
12	1.8	50	65	84

1:250 dilution of infectious juice and various amounts of inhibitor depending upon the dilution or the heat treatment. The heat treatments were carried out by immersing 70 × 5 mm. corked test tubes, containing 1 cc. of the solution, in a water bath held within 0.1° C. of the desired temperature. At the end of the 10-minute treatment, the tubes were immersed in cold water. The total number of lesions

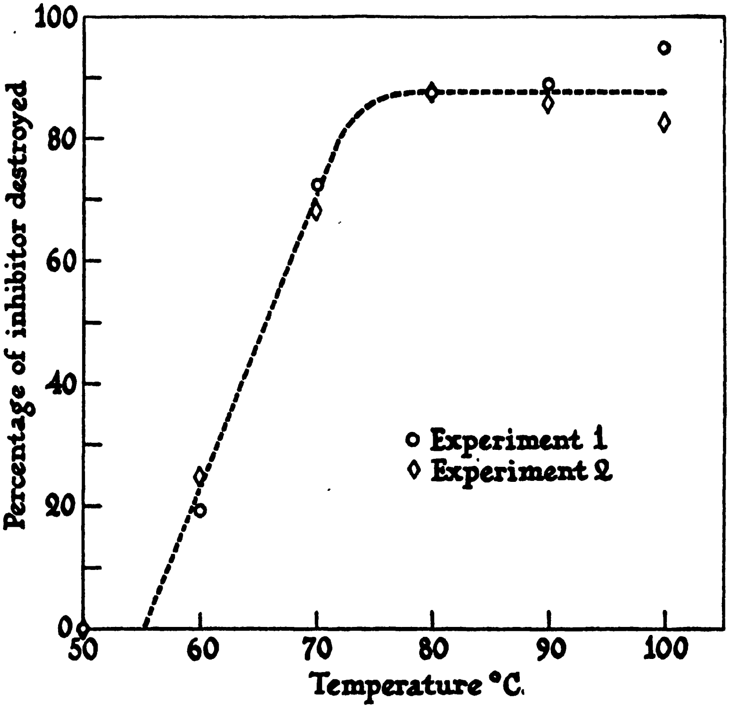

FIG. 4. Inactivation of inhibitor by heating at various temperatures for 10 minutes.

produced by each solution on 33 half-leaves is presented in table 5. By plotting the data for the first 6 solutions—those containing known amounts of inhibitor—in the form of a dilution curve, the data for the other 6 solutions may be interpreted roughly in terms of the amount of inhibitor destroyed by each treatment. The values obtained in this manner are plotted in figure 4. The results of a second experiment, presented in table 5 and figure 4, are in general agreement with those of experiment 1. The inhibitor is apparently readily destroyed by heat.

In order to determine if the infectivity of virus-inhibitor mixtures could be increased by heating, samples having an inhibitor concentration of 5.08 mg. per cc. and a virus concentration 1/3 that of infectious juice were treated at 8 different temperatures between 90° and 55° C. for 10 minutes. After being heated, these samples were diluted 1/3 in buffer to bring the inhibitor content to 1.69 mg. per cc., at which concentration the inhibitor should, if not inactivated by the heat treatment, almost completely suppress lesions. When the samples were compared on bean leaves, it was found that the infectivity increased with the temperature until 70° C. was reached. The infectivity of solutions treated at 75° C. or higher was markedly lower.

Effect of H-ion Concentration on the Inhibitor. In an experiment designed to determine the effect of hydrogen-ion concentration on the inhibitor, 11 concentrated solutions of the inhibitor in distilled water were adjusted to the desired H-ion concentration by the addition of equal volumes of complex buffer mixtures described by Best and Samuel (4). The hydrogen-ion concentrations of the inhibitor solutions were determined immediately and after a period of 24 hours' storage at room temperature. The solutions were then adjusted to approximately pH 7.0 by adding an equal volume of 0.1 M phosphate buffer. In the case of the most alkaline solution and the 2 most acid solutions, a preliminary adjustment with 0.2 N HCl or NaOH was made to bring the solution almost to the neutral point. To each of the neutral solutions was added an equal volume of a 10^{-2} dilution of infectious juice in buffer. These dilutions were calculated to bring the concentration of the inhibitor, if unaltered by the treatment, to 1.8 mg. per cc., an amount that would almost completely prevent infections. The 11 solutions and a control solution to which no inhibitor had been added were compared on bean leaves. The experiment was repeated twice; once with *Agallia constricta* Van Duzee as a source of inhibitor. The total number of lesions produced by each solution on 33 half-leaves is presented in table 6. The experiments show that inhibitor is destroyed in both acid and alkaline solutions. At hydrogen-ion concentrations between pH 5.5 and pH 8.7 the destruction is evidently much slower than in more acid or more alkaline solutions.

Ultrafiltration of Virus-inhibitor Mixtures. Although attempts to remove the inhibitor by dialysis failed, it seemed possible that the inhibitor particles were much smaller than the virus particles and that virus and inhibitor might be separated by ultrafiltration. To test the hypothesis, a virus-inhibitor mixture was prepared containing the infectious juice at a dilution of 1:10 and inhibitor at a concentration of 16.2 mg. per cc. The mixture at pH 7.0 was filtered twice through Hy-flo Standard Super-cel. Ten cc. of the filtrate were then passed under 30-lb. pressure through an Elford membrane, prepared by Thornberry (31), with pores small enough to retain the virus. The precipitate on the membrane was washed by passing 20 cc. of buffer through the membrane. The membrane and the precipitate were ground in 10 cc. of buffer, the coarse material removed by low-speed centrifugation, and the supernatant tested for infectivity at dilutions

TABLE 6

The Effect of Hydrogen-Ion Concentration on the Inhibitor

Inhibitor solution	pH[a]		Lesions produced in 33 half-leaves after virus was added to solutions readjusted to pH 7.0		
	At beginning of storage	At end of 24 hours' storage	Experiment 1	Experiment 2	Experiment 3
1	1.32	1.31	1580	1320	825
2	2.35	2.38	2055	1748	1774
3	3.29	3.43	2655	1377	2663
4	4.21	4.55	1020	672	352
5	5.18	5.46	934	687	217
6	6.13	6.05	799	406	189
7	6.97	6.88	249	260	155
8	8.34	7.57	441	435	235
9	9.12	8.72	718	365	700
10	9.54	9.33	1020	499	1546
11	11.61	11.87	3253	2385	2904
12	No inhibitor and no pH treatment		3068	4188	3294

[a] The pH data are those for experiment 1. The pH data for experiments 2 and 3 were practically the same and are omitted to conserve space.

of 10^0, 10^{-1}, 10^{-2}, and 10^{-3}. For comparison, tests were made with the same dilutions of infectious juice without inhibitor and with the

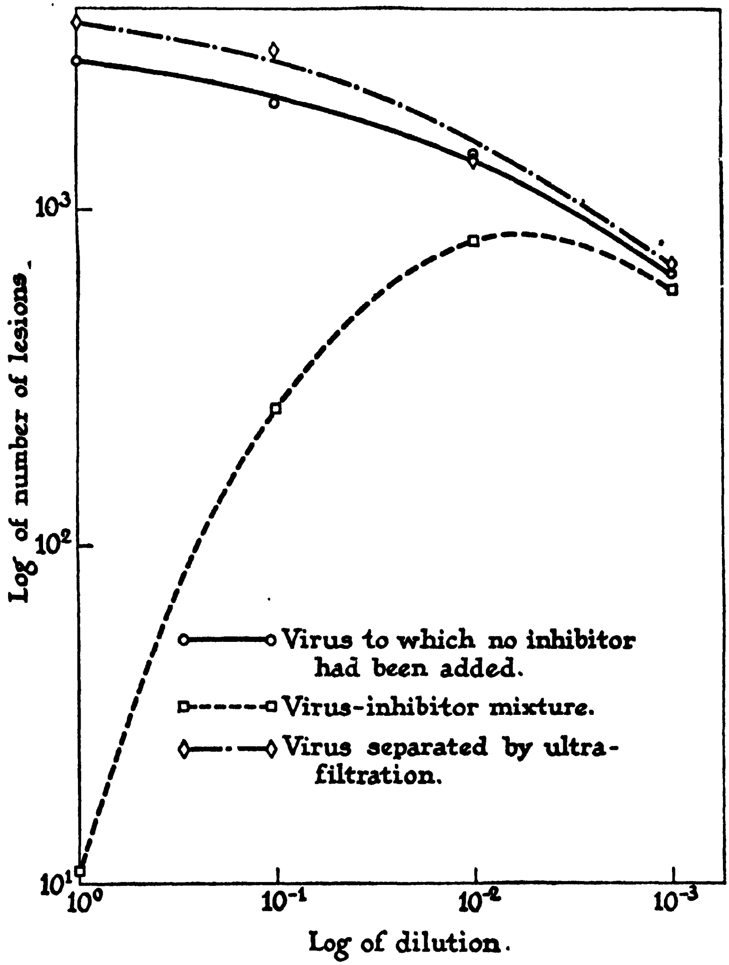

FIG. 5. Dilution curves showing separation of tobacco-mosaic virus and inhibitor by ultrafiltration.

same dilutions of virus-inhibitor mixture, which had not been subjected to ultrafiltration. The lesions produced by each of the 12 solutions on 33 half-leaves are plotted in figure 5. The infectivity of the preparation that had been treated by ultrafiltration was as great as that of the inhibitor-free preparation, indicating that the virus had been freed from detectable amounts of inhibitor. These results were confirmed in a second experiment.

To determine if the inhibitor passed through the membrane, virus was added to the filtrate from the ultrafilter and the infectivity of the mixture tested on bean leaves. The filtrate reduced the infectivity of the virus, thus indicating that the inhibitor passed through the filter.

Ultracentrifugation of Virus-inhibitor Mixtures. It has been shown that the tobacco-mosaic virus can be sedimented by means of the ultracentrifuge (32). The probability that the inhibitor particles were smaller than the virus particles also suggested that a separation of the two might be effected by means of ultracentrifugation. A virus-inhibitor mixture was prepared containing a 10^{-1} dilution of infectious juice and an inhibitor concentration of 16.4 mg. per cc. Two 7-cc. samples of the mixture were ultracentrifuged for 1 hour in a field the mean of which was approximately 45,000 times gravity. The pellet from the first sample was resuspended in its own supernatant; that from the second was resuspended in 7 cc. of fresh buffer. The coarser particles in both samples were then removed by low-speed centrifugation. Each sample was ultracentrifuged again, the pellet resuspended as before, and the larger particles once more removed by low-speed centrifugation. The infectivities of 10^0, 10^{-1}, 10^{-2}, and 10^{-3} dilutions of the two samples were then tested on bean leaves along with corresponding dilutions of virus solution to which no inhibitor had been added. The number of lesions produced by each of the 12 solutions on 33 half-leaves is plotted in figure 6. The infectivity of the sample ultracentrifuged and resuspended in fresh buffer is as great as that of the virus solution alone, while the sample ultracentrifuged and resuspended in its own supernatant shows the dilution curve typical of virus-inhibitor mixtures. The data show that solutions can be freed of detectable amounts of inhibitor by ultracentrifugation. Two repetitions of the experiment gave similar results.

Possible Protein Nature of the Inhibitor. The inhibiting action of normal serum upon the infectivity of the tobacco-mosaic virus was noted by Mulvania (22). Since then the inhibitory action of protein solutions has been studied by a number of workers. The marked inhibitory action of trypsin has been especially studied (19, 8, 26, 9). Several workers (29, 1, 30, 2) have shown that enzymes like trypsin occur in insects. It may be that the inhibitor in insect juice is a trypsin-like substance. The nondialyzable, thermolabile nature of the inhibitor, its instability in acid or alkaline solutions, and the sim-

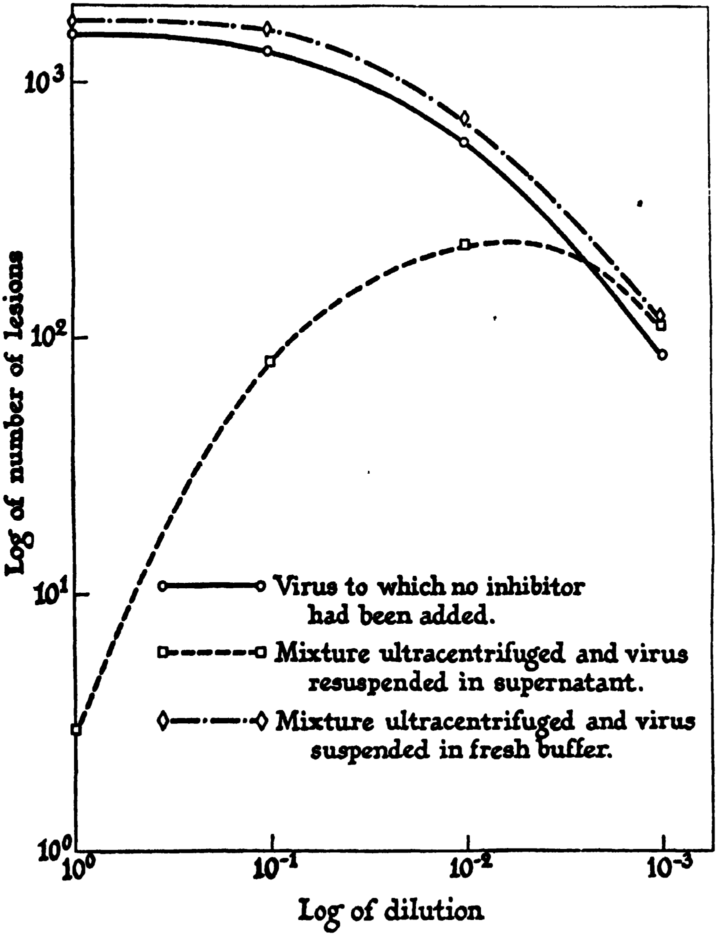

FIG. 6. Dilution curves showing separation of tobacco-mosaic virus and inhibitor by ultracentrifugation.

ilarity of its action to that of proteins suggest that it may be a protein. With this possibility in mind, 3 stock inhibitor solutions were analyzed for protein nitrogen. The solutions analyzed were the same as those used in the experiments in which tests were made of the effect of various concentrations of inhibitor on the virus. The protein nitrogen was determined by a Kjeldahl micro-method described by Folin and Farmer (11) as modified by Northrop (23) but employing selenium oxychloride as a catalyst (18). The stock solutions with an inhibitor concentration of 17.94 mg. per cc. had a protein nitrogen content of from 0.16 to 0.18 mg. per cc. Calculations from data in figure 1 show that an inhibitor concentration causing a 50 per cent reduction in lesions would contain only about 0.0015 mg. of protein nitrogen per cc. However, only a fraction of the protein may be active. These results indicate that if the inhibitor is a protein it is a highly active one.

DISCUSSION

The apparently general inhibiting action of insect juices upon the infectivity of plant viruses would seem to be one reason for the practical absence of reports of successful direct inoculations of plants with juices of viruliferous insects and the failure to employ such inoculations for the study of plant viruses in their insect vectors. The success of McClintock and Smith (20) in inoculating spinach with the spinach-blight virus by pin punctures through juice from viruliferous aphids appears to be the one exception. Smith (24) reported successful inoculation of cowpeas with regurgitated juice or abdominal contents of bean leaf beetles, *Ceratoma trifurcata* Forst., that had fed on plants diseased with cowpea mosaic. This is hardly comparable with the problem under discussion, since his inoculum may not have been very different from freshly expressed juice from diseased plants. The successful transfer of virus to insects by feeding them juice of viruliferous insects (10) or by needle inoculation with the juice (28) gives no indication of the action of the inhibitor in such transfers. It may be that the inhibitor fails to act on the insect cells invaded by the virus.

Caldwell (9) holds that the inhibitory action of trypsin is brought about chiefly by an action upon the virus, while Stanley (26) holds that the principal action is upon the plant. The writer's experiments indicate that the chief action of the inhibitor in insect juices is not upon the virus. The injury of plants by concentrated solutions of inhibitor, its immediate action when added to virus solutions, and the fact that reduction in infectivity depends chiefly on inhibitor concentration and is affected only slightly by virus concentration support this conclusion. The experiments on the dilution, ultrafiltration, and ultracentrifugation of virus-inhibitor mixtures limit any important action between virus and inhibitor to an association of the two that is very readily broken without appreciable injury to the virus. However, the slight increase in the percentage reduction in lesions by a given concentration of inhibitor as virus concentration decreases suggests some action of the inhibitor on the virus. It is conceivable that insect juice might be quite destructive to a virus less stable than the tobacco-mosaic virus.

SUMMARY

Juices of the insect vectors *Aceratagallia sanguinolenta*, *Aedes aegypti*, *Aphis rumicis*, *Eutettix tenellus*, *Macrosiphum pisi*, *M. solanifolii*, *Macrosteles divisus*, and *Myzus persicae* inhibit the infectivity of tobacco-mosaic virus for Early Golden Cluster beans. Juices of macerated clover leaf hoppers inhibit the infectivity of plant juices containing the viruses of potato yellow dwarf, tobacco mosaic, potato X, turnip mosaic, tobacco necrosis, or tobacco ring spot No. 1, when mixtures of insect juice and virus are inoculated on suitable test plants.

The infectivity of certain mixtures of tobacco-mosaic virus and clover leaf-hopper juice can be increased by dilution or by heat treatment.

The inhibitor in clover leaf-hopper juice is thermolabile, not readily dialyzable, and unstable in acid or alkaline solution. If the inhibitor is a protein, as seems probable, it is very active, since 0.15 mg. of clover leaf hoppers, containing only about 0.0015 mg. of soluble protein nitrogen, reduces the infectivity of 1 cc. of a solution of tobacco-mosaic virus by 50 per cent. A given concentration of inhibitor in the presence of different concentrations of tobacco-mosaic virus reduces the number of primary lesions in beans by approximately a constant percentage.

Tobacco-mosaic virus is not destroyed by the inhibitor in clover leaf-hopper juice. The virus and the inhibitor can be separated by subjecting mixtures of the two to either ultrafiltration or ultracentrifugation.

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